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Title of Invention

EFFECTS OF GDNF AND NGF ON SODIUM CHANNELS IN DRG NEURONS

Applicants For DO/EO/US

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(I).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventors (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 14. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☒ A SECOND or SUBSEQUENT preliminary amendment.
14. ☒ Other items or information:
 - a. PCT/IB/308
 - b. PCT/IB/332
 - c. PCT/ISA/210

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15. [X] The following fees are submitted:

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EFFECTS OF GDNF AND NGF ON SODIUM CHANNELS IN DRG NEURONS

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RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Application Serial No.

- 10 09/354,147, filed July 16, 1999, which is a continuation-in-part of PCT International Application No. PCT/US99/02008, also entitled "Modulation of Sodium Channels of Dorsal Root Ganglia," filed January 29, 1999, each of which are related to U.S. Provisional Application 60/072,990, filed January 29, 1998, U.S. Provisional Application 60/109,402 entitled "Modulation of Sodium Channels in Dorsal Root Ganglia", filed
- 15 November 20, 1998 and to U.S. Provision Application 60/109,666, entitled "Differential Role of GDNF and NGF in the Maintenance of Two TTX-Resistant Sodium Channels in Adult DRG Neurons," filed on November 20, 1998, all of which are herein incorporated by reference in their entirety.

20 FIELD OF THE INVENTION

The present invention relates to the role of glial-derived nerve factor (GDNF) and Nerve Growth Factor (NGF) in modulating the activity of tetrodotoxin (TTX)-resistant sodium channels in dorsal root ganglion (DRG) neurons.

25 BACKGROUND

- Small dorsal root ganglion (DRG) neurons give rise to c- and A δ -fibers and are predominantly nociceptive (Lynn and Carpenter, 1982; Kress et al., 1992). Many of these neurons display somatic sodium current components that are relatively resistant to tetrodotoxin (TTX) (Kostyuk et al., 1982; Roy and Narahashi, 1992), and it has been
- 30 suggested that TTX-resistant (TTX-R) sodium currents play an important role in nociceptive transmission (Jeftinija, 1994; Gold et al., 1996). Transcripts for two TTX-R sodium channels, SNS/PN3 and NaN, are preferentially expressed in small DRG neurons (Akopian et al., 1996; Sangameswaran et al., 1996; Dib-Hajj et al., 1998b), and may be

responsible for the TTX-R sodium current observed in the soma (Kostyuk et al., 1981; Bossou and Feltz, 1984; McLean et al., 1988; Caffrey et al., 1992; Roy and Narahashi, 1992) and c-fibers (Quasthoff et al., 1995) of these neurons.

Following transection of the sciatic nerve, TTX-R sodium currents in DRG
5 neurons are attenuated (Rizzo et al., 1995; Cummins and Waxman, 1997) and
concomitantly SNS/PN3 (Dib-Hajj et al., 1996; Okuse et al., 1997) and NaN (Dib-Hajj et
al., 1998b) transcripts are down-regulated. Axotomy prevents the retrograde transport of
nerve growth factor (NGF) from peripheral targets and this may account for many of the
phenotypic changes that appear in DRG neurons following axotomy (for review see Verge
10 et al., 1996). In agreement with a role for NGF in maintaining TTX-R sodium currents,
infusion of NGF to the transected nerve stump restores SNS/PN3 mRNA to near-normal
levels, but only partially rescues TTX-R currents (Dib-Hajj et al., 1998a).

Several mechanisms may explain why NGF only partially restores TTX-R currents
following axotomy; an intriguing possibility, however, is that neurotrophins other than
15 NGF regulate the expression of TTX-R sodium channels in the subpopulation of DRG
neurons that lack receptors for NGF. Glial cell line-derived neurotrophic factor (GDNF)
has been suggested to be important for the maintenance of phenotypic properties in the
subset of small sensory neurons that lack NGF receptors (Molliver et al., 1997; Bennett et
al., 1998b), and intrathecal administration of GDNF can ameliorate the reduction in
20 conduction velocity in small-diameter axons after sciatic nerve transection (Bennett et al.,
1998b). Although a substantial body of evidence demonstrates a role for NGF in the
regulation of specific sodium (Kalman et al., 1990; D'Arcangelo et al., 1993; Zur et al.,
1995) and potassium (Sharma et al., 1993; Lesser and Lo, 1995) channel expression, the
actions of GDNF on ion channel expression has not been established.

25 GDNF-sensitive and NGF-sensitive neurons can be differentiated by their different
ability to bind the lectin IB4 from *Griffonia simplicifolia*. IB4-binding (IB4⁺) neurons
express the receptor/transducing elements necessary to respond to GDNF whereas IB4⁻
neurons generally express the NGF receptors, TrkA and p75 (Averill et al., 1995; Wright
and Snider, 1995; Bennett et al., 1996; Molliver et al., 1997; Bennett et al., 1998b). Based
30 on differences in terminal fields in the spinal cord and certain differences in phenotype of
IB4⁺ and IB4⁻ neurons, it has been suggested that these two subpopulations of small DRG

neurons may play distinct roles in nociceptive transmission (see Snider and McMahon, 1998).

In the present study, the expression of SNS/PN3 and NaN in IB4⁺ and IB4⁻ DRG neurons was examined to determine 1) whether subpopulations of small DRG neurons
5 express distinct TTX-R sodium channels, with different current characteristics and 2) whether NGF and GDNF have differential effects on the expression of SNS/PN3 and NaN, and on TTX-R currents. Inappropriate electrical activity may be involved in some pain syndromes (Matzner and Devor, 1994), and the expression and differential regulation of specific sodium channel gene products in selected sensory neurons may have important
10 implications for pharmaceutical management of pain. Consequently, there is a need for compositions that modulate expression of these genes and also for methods to identify and use the such compositions.

SUMMARY OF THE INVENTION

15 The present invention provides a new means of altering or modulating inappropriate electrical activity which may be involved in pain syndromes. The present invention includes a method to treat pain or hyperexcitability phenomena in an animal or human subject by administering an amount of GDNF or GDNF-related molecule that is effective to alter TTX-R Na⁺ current flow through NaN sodium channels in sensory
20 neurons such as DRG or trigeminal neurons.

The present invention also includes a method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of GDNF or a GDNF-related molecule that is capable of at least partially restoring the normal balance between various types of TTX-R and TTX-S sodium channels in
25 sensory neurons such as DRG or trigeminal neurons.

In another embodiment, the invention includes a method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of modulating the transcription or translation of mRNA encoding sodium channels selected from the group consisting of SNS/PN3 and
30 NaN channels. Such agents includes neurotrophins such as NGF and GDNF.

In another embodiment, the invention includes a method to treat pain, paraesthesia

or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of altering the transcription or translation of mRNA encoding the NaN sodium channel.

Another aspect of the invention includes a method of identifying an agent which modulates TTX-R Na⁺ current through NaN channels comprising the step of determining whether the agent alters or modulates the expression of GDNF or at least one biological activity of GDNF.

Also, the invention includes a method to screen candidate compounds for use in treating pain and hyperexcitability phenomena comprising the steps of exposing the cell to the compound in the presence or absence of GDNF and determining the resultant level of expression or activity of the cell's Na⁺ channels.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

Figure 1. Cell size distribution of DRG neurons positive and negative for SNS/PN3 (A) and NaN mRNA (B). Data for each graph were pooled from four independent experiments by dividing the OD of each neuron by the mean OD of all neurons captured in that experiment. Neurons with a relative intensity > 0.8 are considered positive for SNS/PN3 or NaN. For each size bin, the graphs present the percent of all neurons that are positive and negative for SNS/PN3 (A) or NaN (B). n = 413 for SNS/PN3. n = 263 for NaN. Both SNS/PN3 and NaN are expressed in a large proportion of small DRG neurons.

Figure 2. SNS/PN3 and NaN mRNA in representative IB4⁺ and IB4⁻ DRG neurons. IB4 binding, visualized using biotin-labeled IB4 and Cy2-labeled streptavidin, can be readily recognized after *in situ* hybridization. Examples of 1 day *in vitro* ("DIV") neurons processed for *in situ* hybridization and IB4-binding are shown. A,C) IB4 positivity and negativity after SNS/PN3 *in situ* hybridization. B,D) Corresponding Nomarski images of the same neurons. SNS/PN3 mRNA is expressed in both IB4⁺ (A,B) and IB4⁻ (C,D) DRG neurons. E,G) IB4 binding after NaN *in situ* hybridization. F,H) Corresponding Nomarski images of the same neurons. NaN mRNA is expressed predominantly in IB4⁺ neurons (E, F), whereas NaN was undetectable in many IB4⁻ neurons (G,H). Scale bar is 25 μ m.

Figure 3. SNS/PN3 (A) and NaN (B) distribution in small (<30 μ m) IB4⁺ and IB4⁻

neurons. (A,B) Frequency diagram showing the percentage of IB4⁺ and IB4⁻ neurons, respectively, within each hybridization intensity bin (bin-width 0.4 unit). The percentage of neurons is plotted at the midpoint of each bin. The relative intensity was calculated by subtracting the background intensity and then dividing the optical density of each neurons
 5 by the mean of all neurons examined in that experiment. Data was pooled from four independent experiments. A) SNS/PN3 mRNA is expressed in both in IB4⁺ and IB4⁻ DRG neurons. Note that the most intensely stained cells (relative intensity >2.0) were almost exclusively IB4⁻. The difference in SNS/PN3 hybridization signal between IB4⁺ and IB4⁻ neurons was significant ($p < 0.05$, $n = 338$, Mann-Whitney Rank sum test). B) NaN is
 10 expressed predominantly in IB4⁺ DRG neurons. The difference in NaN hybridization signal between IB4⁺ and IB4⁻ neurons was significant ($p < 0.001$, $n = 242$, Mann-Whitney Rank sum test).

Figure 4. Comparison of sodium currents in IB4⁺ and IB4⁻ DRG neurons. A)
 Families of current traces recorded from representative neurons without TTX in the bath
 15 are shown. While 97% of the IB4⁺ neurons exhibited both fast and slow currents (left panel), only 63% of IB4⁻ neurons exhibited both fast and slow currents (middle panel). 37% of IB4⁻ neurons exhibited predominantly fast currents (left panel). The currents were elicited by 20 ms test pulses to -10 mV after 500 ms prepulses to potentials over the range of -130 mV to -10 mV. The inset graph in each panel shows the corresponding steady-
 20 state inactivation curves for each cell. Current is plotted as a fraction of peak current. Two current components can be easily resolved in the left and middle panels; a slowly inactivating component that has a relatively depolarized voltage-dependence of inactivation (V_h) and a fast inactivating component that has a more negative V_h . The steady-state inactivation curves for these cells are bimodal because of the different
 25 inactivation properties of the two components (arrows indicate point of inflection). The IB4⁻ cell in the right panel, on the other hand, appears to exhibit only fast-inactivating currents and the steady-state inactivation is not inflected. B) The midpoints of steady-state inactivation for the slow current component in IB4⁺ (solid squares, $n=33$) and IB4⁻ F/S (open circles, $n=20$) neurons are plotted as a function of the slow current density. The fast
 30 currents were eliminated using prepulse inactivation. The horizontal dashed lines indicate the average midpoint of inactivation for the slow currents in IB4⁺ neurons (-37.7 ± 1.0 mV)

and IB4⁻ F/S neurons (-31.3 ± 1.1 mV). C) Normalized activation (circles) and steady-state inactivation (squares) curves for the slow currents in IB4⁺ (filled symbols, $n=33$), and IB4⁻ (open symbols, $n=20$) neurons are shown. Cells were held at -100 mV, and prepulsed to -50 mV to inactivate TTX-S currents. Activation was measured with 40 ms test pulses ranging from -70 to $+40$ mV in 5 mV steps and the midpoint of activation was -23.6 ± 1.3 mV for IB4⁺ neurons and -17.4 ± 1.6 mV for IB4⁻ neurons. Steady-state inactivation was measured with 500 ms prepulses and 20 ms test pulses to -10 mV. Error bars indicate standard error.

Figure 5. Comparison of TTX-R currents in IB4⁺ and IB4⁻ neurons. A) Families of voltage-activated TTX-R current traces recorded from representative IB4⁺ and IB4⁻ neurons with 250 nM TTX in the bath are shown. While all of the IB4⁺ neurons exhibited large (>3 nA) TTX-R currents (left panel; $n=30$), 60% of the IB4⁻ exhibited large TTX-R currents (middle panel; $n=18$) and 40% of the IB4⁻ cells exhibited little or no TTX-R current (right panel; $n=12$). The currents were elicited by 200 ms test pulses ranging from -80 to $+40$ mV in 5 mV steps. Cells were held at -100 mV. B) Normalized activation (circles) and steady-state inactivation (squares) curves for the TTX-R currents in IB4⁺ (filled symbols, $n=30$), and IB4⁻ (open symbols, $n=18$) neurons are shown. Error bars indicate standard error. Cells were held at -100 mV and activation was measured with 200 ms test pulses ranging from -80 to $+40$ mV in 5 mV steps. Steady-state fast inactivation was measured with 500 ms prepulses and 20 ms test pulses to -10 mV. C) Scatterplots showing the midpoint of steady-state inactivation against the midpoint of activation for TTX-R currents in IB4⁺ (filled squares) and IB4⁻ (open circles) neurons. The boxes delineate the mean \pm standard deviation for the IB4⁺ (solid outline) and IB4⁻ (dashed outline) data.

Figure 6. Effects of neurotrophins on the expression of SNS/PN3 mRNA. The optical densities of neurons captured from three separate experiments were normalized and pooled. The graph represents the mean normalized OD for IB4⁺ and IB4⁻ neurons respectively for each condition. Error bar represents standard error. All treatments produced a significant difference in the respective subpopulations ($p_c < 0.01$; Bonferroni t test for multiple comparisons), compared to 7 DIV control neurons. Whereas GDNF was significantly ($p_c <$

0.001) more effective on IB4⁺ neurons compared to IB4⁻ neurons, no significant difference ($p_c = ns$) was observed between IB4⁺ and IB4⁻ neurons after NGF treatment.

Figure 7. SNS/PN3 *in situ* hybridization of representative IB4⁺ and IB4⁻ DRG neurons after treatment with NGF and GDNF for 7 days. Without addition of either growth factor, little hybridization signal is seen in either IB4⁺ (A) or IB4⁻ (B) neurons. NGF increases the signal in both IB4⁺ (C) and IB4⁻ (D) neurons. GDNF increased the hybridization signal predominantly in IB4⁺ neurons (E), with little effect on IB4⁻ neurons (F). Scale bar is 25 μ m.

Figure 8. The upregulation of SNS/PN3 mRNA by NGF is blocked by K252a. The diagram shows the relative SNS/PN3 signal in neurons treated with NGF alone (50 ng/ml; $n=309$), or in combination with K252a at 100nM ($n=151$), 200nM ($n=248$) or 400nM ($n=65$). Each bar represents the mean of the pooled, normalized optical densities from 2 to 4 independent experiments. Error bars represent standard error. K252a alone does not affect the SNS/PN3 hybridization signal intensity at these concentrations (Control: $n=216$; 100nM K252a: $n=156$; 400nM K252a: $n=86$). *The SNS/PN3 hybridization signal was significantly ($p_c < 0.001$; Bonferroni *t* test) less intense in neurons treated with both NGF and K252a compared to neurons treated with NGF alone.

Figure 9. Effects of neurotrophins on the expression of NaN mRNA. Data from three different experiments are normalized and pooled. The graph represents the mean normalized OD for IB4⁺ and IB4⁻ neurons respectively for each condition. Error bar represents standard error. At 7 DIV the NaN hybridization signal was significantly reduced in IB4⁺ neurons. GDNF increased the NaN hybridization signal in IB4⁺ neurons, but had no effect on in IB4⁻ neurons. NGF did not increase NaN expression in either IB4⁺ or IB4⁻ neurons. *Significantly different from 7 DIV control by Bonferroni *t* test for multiple comparisons ($p_c < 0.001$).

Figure 10. NaN *in situ* hybridization of IB4⁺ and IB4⁻ DRG neurons after treatment with NGF and GDNF for 7 days. Without addition of either growth factor, little hybridization signal is seen in either IB4⁺ (A) or IB4⁻ (B) neurons. NGF did not increase the signal in either IB4⁺ (C) or IB4⁻ (D) neurons. GDNF increased the hybridization signal markedly in IB4⁺ neurons (E), but had no effect on IB4⁻ neurons (F). Scale bar is 25 μ m.

Figure 11. GDNF increases TTX-R currents in cultured DRG neurons. A)

Families of voltage-activated TTX-R current traces recorded from representative DRG neurons. Currents from control, NGF-treated, GDNF-treated and GDNF+NGF-treated neurons are shown. Cells were studied after 7 DIV. The bath solution contained 250 nM TTX. The currents were elicited by 200 ms test pulses ranging from -80 to +40 mV in 5 mV steps. Cells were held at -100 mV. B) TTX-R peak current amplitude from control, NGF-treated, GDNF-treated and GDNF+NGF-treated neurons after 7DIV are shown. For comparison, the TTX-R peak current amplitude measured at 1 DIV is also shown. GDNF treatment significantly increases the size of the TTX-R current compared to the control 7 DIV neurons.

- 10 *Figure 12.* Figure 12A shows families of voltage-activated TTX-R current traces recorded from representative SNS-null neurons after 1 day in vitro (1DIV), untreated SNS-null neurons after 7 days in vitro (1DIV) and GDNF-treated SNS-null neurons after 7 days in vitro (DIV). Figure 12B shows TTX-R peak current amplitude from 1DIV, untreated 7DIV, GDNF-treated 7DIV and NGF-treated 7DIV SNS-null neurons.
- 15 *Figure 13.* Figure 13A shows that GDNF administration attenuates the decrease in current density that results from axotomy. Figure 13B shows that GDNF administration attenuates the decrease in persistent Na⁺ currents that results from axotomy.

DETAILED DESCRIPTION

Following axotomy, electrophysiological properties of small dorsal root ganglion (DRG) neurons are markedly altered, with attenuation of TTX-R sodium currents and the appearance of rapidly repriming TTX-S currents. The reduction in TTX-R currents has
5 been attributed to a down-regulation of sodium channels SNS/PN3 and NaN. While infusion of exogenous NGF to the transected nerve restores SNS/PN3 transcripts to near-normal levels in small DRG neurons, TTX-R sodium currents are only partially rescued. Binding of the isolectin IB4 distinguishes between two major subpopulations of small DRG neurons: IB4⁺ neurons, which express receptors for the GDNF family of
10 neurotrophins, and IB4⁻ neurons that predominantly express trkA.

The present inventors have shown that SNS/PN3 is expressed in approximately one-half of both IB4⁺ and IB4⁻ DRG neurons, while NaN is preferentially expressed in IB4⁺ neurons. Whole-cell patch-clamp studies demonstrate that TTX-R sodium currents in IB4⁺ neurons have a more hyperpolarized voltage-dependence of activation and
15 inactivation than do IB4⁻ neurons, suggesting different electrophysiological properties for SNS/PN3 and NaN. Utilizing an *in vitro* model of axotomy, it was confirmed that NGF restores SNS/PN3 mRNA levels and demonstrate that the trk antagonist K252a blocks this rescue, indicating a central role for trkA receptors in the signaling pathway. The down-regulation of NaN mRNA is, nevertheless, not rescued by NGF-treatment in either IB4⁺ or
20 IB4⁻ neurons and NGF-treatment does not significantly increase the peak amplitude of the TTX-R current in small DRG neurons *in vitro*. In contrast, GDNF-treatment causes a two-fold increase in the peak amplitude of TTX-R sodium currents and restores both SNS/PN3 and NaN mRNA to near-normal levels in IB4⁺ neurons. These observations provide a mechanism for the partial restoration of TTX-R sodium currents by NGF in axotomized
25 DRG neurons, and demonstrate that the neurotrophins NGF and GDNF differentially regulate sodium channels SNS/PN3 and NaN.

A. TTX-R sodium channels in IB4⁺ and IB4⁻ DRG neurons

Several groups have suggested that DRG neurons exhibit at least two distinct types
30 of TTX-R currents based on the voltage-dependence of activation and inactivation (Brau and Elliott, 1998; Rush et al., 1998; Scholz et al., 1998). The present inventors' results

indicate that NaN and SNS/PN3 could underlie these distinct TTX-R currents in small DRG neurons. Expression of SNS/PN3 in *Xenopus* oocytes gives rise to voltage-gated sodium currents with slow kinetics and resistance to high concentrations of TTX (Akopian et al., 1996; Sangameswaran et al., 1996). NaN, while not as yet heterologously expressed, is predicted to be TTX-R based on sequence analysis (Dib-Hajj et al., 1998b). Whole-cell patch-clamp recordings of IB4⁺ and IB4⁻ neurons revealed that the TTX-R currents show a voltage-dependence of steady-state activation and inactivation that is hyperpolarized in IB4⁺ neurons. While two-thirds of IB4⁻ cells express TTX-R currents, only about a third express NaN mRNA. Thus, SNS/PN3 may underlie much of the slow TTX-R current that is observed in IB4⁻ cells. In support of this speculation, SNS/PN3 channels expressed in *Xenopus* oocytes give rise to slow TTX-R currents with a midpoint of inactivation of -30 mV (Akopian et al., 1996), which is similar to what it was observed in the majority of IB4⁻ cells. Conversely, although all IB4⁺ neurons display slow TTX-R currents, only about one half express SNS/PN3 mRNA. Since NaN is expressed predominantly in IB4⁺ neurons, NaN may account for much of the slow TTX-R current that is observed in IB4⁺ cells. Based on these observations, it is not unreasonable to conclude that NaN corresponds to a TTX-R sodium channel with a more negative midpoint of inactivation than SNS/PN3 and a lower threshold for activation.

The present inventors' observations that NaN is expressed in about 70% of small IB4⁺ neurons, and that SNS/PN3 mRNA is present in slightly more than 50% of both IB4⁺ and IB4⁻ small neurons, indicates that NaN and SNS/PN3 are coexpressed in some of the cells. It would be predicted, therefore, that some neurons would express ensembles of NaN and SNS/PN3 currents and hence the electrophysiological analysis of IB4⁺ and IB4⁻ cells would underestimate the difference between the two channels. Coexpression of two slow TTX-R channels with subtly different voltage dependencies could in part account for the interneuronal variation that has been described for slow TTX-R currents in small DRG neurons (Rizzo et al., 1994). Functionally, nociceptive neurons might fine-tune their integrative and repetitive firing properties by altering the relative expression of SNS/PN3 and NaN channels. These channels may also differ in other properties, such as subcellular localization and sensitivity to second messenger modulation, which could also be important determinants of transductive and/or encoding characteristics of different DRG

neurons.

Most IB4⁺ neurons expressed slow TTX-R currents that were similar to the TTX-R2 currents described by Rush et al. (1998); on the other hand, the TTX-R1 currents were similar to the predominant TTX-R current in IB4⁻ cells. Fast TTX-R currents such as those described by Scholz et al. (1998) in either IB4⁺ or IB4⁻ cells were not observed. In the IB4⁻ group, about one-third of the small neurons had very low amplitude, or no TTX-R currents, suggesting that this group of neurons expressed neither SNS/PN3 nor NaN. This subpopulation of IB4⁻ neurons, which expressed relatively large fast TTX-S currents, may represent a distinct subset of sensory neurons.

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B. Effect of neurotrophins on SNS/PN3 and NaN mRNA and TTX-R sodium currents

NGF has previously been shown to play a prominent role in the regulation of sodium channel/current expression in PC12 cells, as well as DRG neurons. In PC12 cells, NGF up-regulates sodium channels II and PN1 through distinct signal transduction pathways, with the latter being Ras-independent (D'Arcangelo et al., 1993). Interestingly, short-term (1-minute) application of NGF up-regulates PN1 but not brain type II in PC12 cells (Toledo-Aral et al., 1995). These observations point to divergent signaling pathways for two distinct TTX-S sodium channels (Noda et al., 1986; Klugbauer et al., 1995). Similar mechanisms may regulate specific sodium channel isoforms in DRG neurons. Consistent with this idea, NGF application accelerates the diversity and acquisition of sodium currents in neonatal DRG neurons (Omri and Meiri, 1990) and increases the threshold for spike generation in young post-natal DRG neurons (Aguayo and White, 1992).

The role of NGF in the regulation of SNS/PN3 mRNA in DRG neurons has been the focus of several studies. Administration of exogenous NGF increases the levels of SNS/PN3 mRNA both *in vitro* and *in vivo* (Black et al., 1997; Dib-Hajj et al., 1998a; this study). Moreover, enhanced levels of tissue NGF in the receptive fields of DRG neurons in carrageenan-induced inflammation are associated with up-regulation of SNS/PN3 (Tanaka et al., 1998), while, in contrast, depleted levels of NGF *in vivo* are accompanied by a down-regulation of SNS/PN3 (Fjell et al., 1999b). These observations point to an

important modulatory role for NGF in SNS/PN3 expression. In contrast, utilizing differing methodological and model systems, Okuse et al. (1997) have provided data suggesting a more limited role for NGF in SNS/PN3 expression. However, in the present study, the present inventors have confirmed the earlier observation that NGF up-regulates SNS/PN3 to near-normal levels in an *in vitro* model of axotomy, and have extended these results to show that the action of NGF on SNS/PN3 expression is not limited solely to IB4⁻ neurons, but can also be detected in IB4⁺ neurons. The NGF-induced up-regulation of SNS/PN3 was blocked by the trk antagonist K252a (Kase et al., 1987), suggesting that this modulatory action is mediated through a direct effect on the DRG neurons. Since a subpopulation of IB4⁺ neurons express trkA (Bennett et al., 1998b), the increase in SNS/PN3 mRNA seen in IB4⁺ neurons after treatment with NGF may reflect an effect on those neurons that also express trkA. NGF, however, does not regulate the expression of all sodium channel mRNAs in DRG neurons that express TrkA receptors, as NGF had no detectable effect on NaN mRNA expression.

While GDNF has well-established roles as a potent survival factor for certain classes of neurons (Lin et al., 1993; Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995) and as a protector of neurons from injury (Tomic et al., 1995; Beck et al., 1995), the effect of this neurotrophin on the electrical properties of neurons is largely unknown. It has been previously shown that intrathecal administration of GDNF ameliorates the reduction in conduction velocity of c-type fibers that follows axotomy (Bennett et al., 1998b), although it is unclear what mechanism is responsible for the enhancement of conduction velocity. The present inventors' work demonstrate here for the first time that GDNF modulates sodium channel expression, upregulating both SNS/PN3 and NaN transcripts. The action of GDNF was substantially more pronounced in IB4⁺ neurons than in IB4⁻ neurons. Since almost all IB4⁺, but few IB4⁻, neurons express receptors for the GDNF-family of neurotrophins (Bennett et al., 1998b), these findings are consistent with an action of GDNF only on those neurons that express the receptor/transducer complex for GDNF.

NGF and GDNF share several characteristics as target-derived, retrogradely-transported neurotrophic factors. In the adult, NGF is primarily expressed in the skin; the levels of NGF are increased in inflamed tissues, and both endogenous and exogenous

increase of tissue NGF levels are associated with pain/hyperalgesia (Lewin et al., 1994; Woolf, 1996; Woolf et al., 1996; Dyck et al., 1997). Conversely, NGF-deprivation *in vivo* prevents hypersensitivity and results in thermal hypoalgesia (Chudler et al., 1997; Bennett et al., 1998a). In contrast to NGF, GDNF is produced at very low levels in adult skin and spinal cord, but is expressed by Schwann cells in the sciatic nerve (Nosrat et al., 1996; Widenfalk et al., 1997), suggesting that Schwann cells may be the primary source of GDNF for adult DRG neurons. It has been suggested that an up-regulation of GDNF in the injured nerve may play an important role for regeneration of sensory neurons following axotomy (Trupp et al., 1995; Hammarberg et al., 1996; Naveilhan et al., 1997; Bär et al., 1998;). Unlike the pain-inducing effect of NGF, McMahon and coworkers have suggested that GDNF may not induce pain (Bennett et al., 1998b). This hypothesis is intriguing in light of the observations of divergent central projections of IB4⁺ and IB4⁻ neurons (Molliver et al., 1995), and the suggestion that the former neurons are critically important in neuropathic pain and the latter in inflammatory pain (see Snider and McMahon 1998). The finding that NGF increases the expression of SNS/PN3, but not NaN mRNA, might suggest that some inflammatory syndromes could arise from an imbalance in sodium channels reflecting different responses to different neurotrophic factors. As noted above, SNS/PN3 may encode a TTX-R sodium current with a more depolarized inactivation curve and a higher threshold for activation. Changes in the levels of NGF and GDNF might therefore affect the electrogenic properties of some small DRG neurons after nerve injury, possibly contributing to the development of hyperexcitability.

C. *Methods of screening for agents to alter or modulate Sodium channel expression or activity.*

Several approaches can be used to identify agents that are able to alter or modulate the GDNF induced Na⁺ current through the SNS/PN3, NaN or other sodium channels. As used herein, "alter" refers to up- or down-regulating the levels or activity of NaN, such as current flow. In general, to identify such agents, a model cultured cell line that expresses the NaN sodium channel and GDNF or a GDNF receptor is utilized, and one or more conventional assays are used to measure Na⁺ current. Such conventional assays include, for example, patch clamp methods, the ratiometric imaging of [Na⁺]_i, and the use of ²²Na

and ^{86}Rb . Alternatively, the amount of NaN RNA or protein may be directly measured by conventional assays such as hybridization of immunoblot assays.

In one embodiment of the present invention, to evaluate the activity of a candidate compound to modulate Na^+ current, an agent is brought into contact with a suitable transformed host cell that expresses a functional GDNF receptor and NaN or GDNF. Cells that express endogenous NaN are also useful for screening of candidate agents. After mixing or appropriate incubation time, the Na^+ current is measured to determine if the agent inhibited or enhanced Na^+ current flow. If the cell line is engineered to express a functional GDNF receptor, the agent to be tested may be brought into contact with a suitable host cell in the presence or absence of exogenously supplied GDNF. Agents that inhibit or enhance Na^+ current are thereby identified.

The preferred agents that alter or modulate the levels or activity of NaN preferably will be selective for the NaN Na^+ channel, may be selective for GDNF or may alter or modulate the GDNF mediated induction of NaN. Similar analyses may be conducted by the skilled artisan to identify agents that alter the effect of other neurotrophic factors. For example, Mildbrandt, J. *et al.* (Neuron, vol. 20, 245-253) describes the discovery of a third member of the GDNF family, Persephin, and reviews the literature on this family. The other two members are: GDNF (the prototype) and nurturin (NTN). Four receptors for these ligands have been identified. See also the March 29th, 1996 issue of the journal: Philosophical Transactions of the Royal Society of London, B. Biological Sciences (philos. trans. R. Soc. Lond. B. Biol. Sci. 1996) which contains multiple chapters on the NGF family of neurotrophic factors and their role in various models.

Agents of the invention may be totally specific (like tetrodotoxin, TTX, which inhibits sodium channels but does not bind to or directly effect any other channels or receptors), or relatively specific (such as lidocaine which binds to and blocks several types of ion channels, but has a preference for sodium channels). Total specificity is not required for an inhibitor or enhancer to be effective; the ratio of its effect on sodium channels vs. other channels and receptors, will determine its effect; and effects on several channels, in addition to the targeted one, may be of interest.

It is contemplated that modulating agents of the present invention can be, as

examples, peptides, small molecules, naturally occurring or synthetic toxins and vitamin derivatives, as well as carbohydrates. A skilled artisan will readily recognize that there is no limit as to the structural nature of the modulating agents of the present invention. It is contemplated that the screening of libraries of molecules will reveal agents that modulate

5 NaN or current flow through it. One class of agents of the present invention are peptide agents whose amino acid sequences are chosen based on the amino acid sequence of the of the NaN Na⁺ channel, of GDNF or a functional GDNF receptor. Such peptide fragments can be routinely identified by exposing a transformed host cell to these agents and measuring any resultant changes in Na⁺ current. Similarly, naturally occurring toxins

10 (such as those produced by certain fish, amphibians and invertebrates) can be screened.

D. Methods of treating pain, paraesthesia or hyperexcitability phenomena.

Agents of the invention may be administered to a human or animal subject. As used herein, a subject can be any mammal, so long as the mammal is in need of

15 modulation of a pathological or biological process mediated by the alteration or modulation of sodium channels such as NaN. The term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

Pathological processes refer to a category of biological processes which produce a

20 deleterious effect. For example, alteration or the modulation of the amount or of a biological activity of NaN may be associated with pain, paraesthesia or hyperexcitability phenomena. As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For instance, pain, paraesthesia or hyperexcitability phenomena may be prevented, altered or modulated by the

25 administration of agents which reduce, enhance or modulate in some way GDNF induction of NaN.

The agents of the present invention can be provided alone, or in combination with other agents that modulate a particular pathological process. For example, an agent of the present invention, such as GDNF, a GDNF-related molecule such as a GDNF peptide, or a

30 NaN peptide can be administered in combination with another agent that alters or modulates Na⁺ current. As used herein, two agents are said to be administered in

combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes.

5 Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The present invention further provides compositions containing one or more agents which modulate expression or at least one activity of a protein of the invention. While
10 individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 0.1 to 100 $\mu\text{g/kg}$ body wt. The preferred dosages comprise 0.1 to 10 $\mu\text{g/kg}$ body wt. The most preferred dosages comprise 0.1 to 1 $\mu\text{g/kg}$ body wt.

In addition to the pharmacologically active agent, the compositions of the present
15 invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition,
20 suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or
25 dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic
30 administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules,

pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

10

EXAMPLES

GENERAL METHODS

1. Cell culture

Adult female Sprague-Dawley rats were deeply anesthetized with xylazine/ketamine (40/2.5 mg/kg; i.p.) and decapitated. The L4 and L5 ganglia were quickly removed and desheathed in sterile complete saline solution (CSS) (pH 7.2). The DRGs were then enzymatically digested for 20 min with collagenase A (1mg/ml; Boehringer-Mannheim, Indianapolis, IN) in CSS and for 15 min. with collagenase D (1mg/ml; Boehringer-Mannheim) containing papain (30 units/ml, Worthington Biochemical Corporation, Lakewood, NJ) in CSS at 37°C. The DRGs were gently centrifuged (100g for 3 min.) and the pellet triturated in DRG-media (DMEM:F12, 10% FCS) containing 1mg/ml bovine-serum albumin (BSA, Fraction V) (Sigma, St. Louis, MO) and 1 mg/ml trypsin inhibitor (Sigma). The cells were then plated on poly-ornithine/laminin-coated glass coverslips and incubated at 37°C in a humidified 95% air /5% CO₂ incubator.

2. Growth factors

To study the effects of GDNF, NGF and brain-derived neurotrophic factor (BDNF) on SNS/PN3 and NaN mRNA expression, and TTX-R sodium currents, cells were treated with DRG media or DRG media supplemented with NGF (50 ng/ml, mouse 7S NGF, Sigma), GDNF (human recombinant, 50 ng/ml, Calbiochem, San Diego, CA), BDNF

(10ng/ml, Regeneron) or DRG media supplemented with a combination of NGF (50 ng/ml) and GDNF (50 ng/ml). The cells were maintained in culture for seven days and one-half of the media was replaced daily. For each experiment a control DRG culture containing neurons derived from L4/L5 ganglia was established and maintained for one
5 day *in vitro* (1 DIV). In some experiments the trk inhibitor K252a was used to determine if the effect of NGF on SNS/PN3 mRNA expression is mediated through the trkA pathway. K252a (Calbiochem) was dissolved in DMSO (1mg/ml) and added to cultures in concentrations ranging from 100 nM - 400 nM.

10 3. *In situ hybridization*

The expression of SNS/PN3 and NaN mRNA in individual neurons was determined by *in situ* hybridization as previously described (Black et al., 1996; Dib-Hajj et al., 1998b). In short, coverslips from the different experimental groups were fixed for 10 min. in 4% formaldehyde in 0.14M Sorensens buffer, pH 7.2, washed several times with
15 diethylpyrocarbonate (DEPC) -treated PBS and permeabilized with 0.1 % Triton X-100 in PBS for 15 min. The coverslips were then rinsed with 2X SSC, prehybridized for 30 min and then hybridized at 58°C overnight using riboprobes (0.25-0.5 ng/ml) specific for SNS/PN3 or NaN. The coverslips were sequentially incubated in 4X SSC, 2X SSC, RNase A (20mg/ml; Sigma; 37°C, 30 min.) and finally 0.2X SSC at 58°C for 3 X 20 min.
20 The coverslips were then blocked with 2% normal sheep serum and 1 % BSA for 20 min and incubated with alkaline phosphates-conjugated anti-digoxigenin F'ab fragments (1:500, Boehringer-Mannheim) overnight at 4°C. Following multiple rinses, the hybridization signal was visualized using NBT histochemistry. Coverslips for each condition in each experiment were kept in the NBT solution for the same length of time;
25 the NBT reaction was monitored visually and stopped before the signal reached saturation.

For co-localization of SNS/PN3 or NaN mRNA with IB4 reactivity, biotin labeled-isolectin B4 (IB4) (40µg/ml, Sigma) was added to the culture medium and incubated for 30 min. at 37°C prior to beginning the *in situ* hybridization protocol. The coverslips were then washed with CSS and *in situ* hybridization was performed as described above with
30 minor modifications. Following hybridization and stringent washes, the coverslips were incubated with streptavidin-Cy2 (40µg/ml, Amersham Life Science Inc, Arlington

Heights, IL) and alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500, Boehringer-Mannheim) in Tris-buffered blocking solution (1% BSA, 2% normal goat serum) overnight at 4°C.

4. Quantification and data analysis

5 The coverslips were examined with a BioRad MRC-600 confocal microscope equipped with brightfield and BHS filter or with a Leica Aristoplan microscope. IB4 reactivity was determined visually by the presence of extracellular fluorescent signal above background levels. Microdensitometric quantification of the SNS/PN3 and NaN hybridization signal was performed as previously described (Black et al., 1997). Briefly, 10 optical density (OD) measurements of the neurons were obtained using the Scion image analysis program. The brightfield gray levels were linearly ($R^2 > 0.99$) calibrated to optical density using optical filters with OD = 0.1, 0.3 and 0.6. All hybridization signals measured were within the linear calibration range. Samples for analysis were obtained from each coverslip by arbitrarily scrolling the coverslip from the upper left quadrant and 15 capturing the first twenty to fifty fields containing distinguishable neurons. The neurons in the captured images were outlined and the area and mean optical density of each cell was determined. To permit pooling of data from different experiments the optical densities were normalized by dividing the OD of each neuron by the mean SNS/PN3 or NaN optical density of the control cells at 1 DIV, processed in the same *in situ* 20 hybridization experiment. In experiments that colocalized SNS/PN3 or NaN mRNA with IB4 binding, the OD of each IB4⁺ or IB4⁻ neuron was normalized as described above using the mean optical density of all (IB4⁺ and IB4⁻) neurons. Neurons were considered positive for SNS/PN3 or NaN if the relative intensity was > 0.8 , which corresponds to a lightly stained neuron. In experiments with K252a added to the medium, the mean OD of all 25 neurons from cultures that had not received any supplement was used to normalize the experimental values.

The Mann-Whitney Rank sum test was used to test if statistically significant differences exist in the expression of SNS/PN3 and NaN mRNA in IB4⁺ and IB4⁻ neurons. To determine if the effects of the different neurotrophins on SNS/PN3 and NaN mRNA 30 expression in IB4⁺ and IB4⁻ neurons were statistically significant, the means of the normalized optical densities pooled from 3 separate experiments were analyzed using a

one-way ANOVA. If a significant difference was detected, a two-tailed Student *t* test was used and the resulting *p*-value was corrected by multiplying by the number of comparisons made (Bonferroni *t* test). Significance was assessed as $p_{\text{corrected}} (p_c) < 0.05$.

5 5. Whole-cell Recordings

Sodium currents were recorded from DRG neurons in the whole-cell patch-clamp configuration 18-30 hrs. after dissociation and plating (1DIV) or after treatment with growth factors for seven days *in vitro* (7 DIV). Prior to recording, the cells were incubated for 30-60 minutes with FITC-labeled Isolectin B4 (40 μ g/ml, Sigma). All recordings were made with an EPC-9 amplifier, a Macintosh Quadra 950 and the Pulse program (v 7.52, HEKA Electronic, Germany). Recording electrodes (0.8-2 M Ω) were fabricated from 1.65-mm capillary glass (WPI) using a Sutter P-87 puller. Cells were not considered for analysis if the initial seal resistance was less than 1 G Ω or if they had high leakage currents (holding current > 1 nA at -80 mV) or an access resistance greater than 5 M Ω . The average access resistance was 2.3 ± 0.8 M Ω (mean \pm standard deviation, *n*=310). Voltage errors were minimized using 70-80% series resistance compensation. Linear leak subtraction and capacitance artifact cancellation were used for all recordings. Membrane currents were filtered at 2.5 KHz and sampled at 10 KHz. The pipette solution contained (in mM): 140 CsF, 2 MgCl₂, 1 EGTA, and 10 Na-HEPES (pH 7.3). The standard extracellular solution contained (in mM): 140 NaCl, 3 KCl, 2 MgCl₂, 1 CaCl₂, 0.1 CdCl₂, and 10 HEPES (pH 7.3). Cadmium was included to block calcium currents. The osmolarity of the solutions was adjusted to 310 mosM (Wescor 5550 osmometer). The liquid junction potential for these solutions was <7 mV; data were not corrected to account for this offset. The offset potential was zeroed before patching the cells and checked after each recording for drift. All recordings were conducted at room temperature (~22° C).

Example 1: SNS/PN3 and NaN mRNA in IB4⁺ and IB4⁻ DRG neurons

Both SNS/PN3 and NaN mRNA are suggested to encode TTX-R sodium channels in DRG neurons (Akopian et al., 1996; Sangameswaran et al., 1996; Dib-Hajj et al.,

1998b), and both are preferentially expressed in small ($< 30 \mu\text{m}$) DRG neurons (Fig. 1). Small DRG neurons have been differentiated into two major subpopulations on the basis of their ability to bind to the lectin IB4 (Averill et al., 1995; Wright and Snider, 1995; Molliver et al., 1997; Bennett et al., 1998b). To establish if SNS/PN3 and NaN are

5 differentially expressed in IB4⁺ and IB4⁻ neurons, localization of SNS/PN3 or NaN mRNA by *in situ* hybridization was combined with IB4 cytofluorescent labeling. IB4 binding was determined by the presence of a clearly identifiable extracellular staining of the soma (Fig. 2). SNS/PN3 mRNA was expressed in both IB4⁺ and IB4⁻ neurons, but neurons with the greatest hybridization signal for SNS/PN3 mRNA were predominantly IB4⁻. The

10 difference in SNS/PN3 mRNA expression between IB4⁺ and IB4⁻ neurons was significant ($p < 0.05$, Mann-Whitney rank sum test) (Fig. 3a). Fifty-two percent of small IB4⁺ neurons ($n=157$) and 64% of small IB4⁻ neurons ($n=226$) expressed SNS/PN3 mRNA. In contrast to SNS/PN3, NaN was expressed predominantly in IB4⁺ neurons ($p < 0.0001$, Mann-Whitney rank sum test) (Fig. 3b.), with 72% of small IB4⁺ neurons ($n=144$) and

15 only 38% of small IB4⁻ neurons ($n=98$) expressing NaN mRNA. These results demonstrate that SNS/PN3 and NaN are differentially expressed in IB4⁺ and IB4⁻ DRG neurons; moreover, the distribution patterns indicate that SNS/PN3 and NaN must be coexpressed in a substantial subpopulation of IB4⁺ DRG neurons, and possibly some IB4⁻ neurons.

20

Example 2: *TTX-R currents in IB4⁺ and IB4⁻ neurons*

Since SNS/PN3 and NaN have different patterns of expression in small IB4⁺ and IB4⁻ neurons, whole-cell patch-clamp recordings from small IB4⁺ and IB4⁻ (17-32 μm diam.) DRG neurons were performed to determine if differences exist in voltage-

25 dependence and kinetic properties of sodium currents in these cells. The neurons were maintained in culture for less than 30 hrs and binding to IB4 was determined prior to recording, permitting us to analyze approximately the same number of IB4⁺ and IB4⁻ neurons. Both fast inactivating ("fast", $\tau_h < 1 \text{ msec}$ at 0 mV) and slow inactivating ("slow", $\tau_h > 2.5 \text{ msec}$ at 0 mV) sodium currents were observed in small DRG neurons.

30 The fast and slow sodium currents observed in the present study were similar to those previously described in small DRG neurons (Caffrey et al., 1992; Roy and Narahashi,

1992; Elliott and Elliott, 1993; Rizzo et al., 1994; Cummins and Waxman, 1997). All IB4⁺ neurons (n=33) analyzed expressed slow sodium currents, and all but one of these IB4⁺ cells also expressed fast sodium currents. In contrast, only 63% of the IB4⁻ neurons (n=32) expressed slow currents, but all IB4⁻ neurons expressed fast currents. Based on
5 these observations, neurons were assigned to one of three groups: IB4⁺ neurons with both fast and slow currents (IB4⁺ F/S), IB4⁻ neurons with both fast and slow currents (IB4⁻ F/S), and IB4⁻ neurons with only fast currents (IB4⁻ F) (Fig. 4A).

Prepulse-inactivation was used (McLean et al., 1988; Roy and Narahashi, 1992; Elliott and Elliott, 1993; Cummins and Waxman, 1997) to separate fast and slow sodium
10 current components in the cells. The peak amplitudes for fast and slow sodium current components are compared in Table 1. The slow current peak amplitude was similar for IB4⁺ F/S and IB4⁻ F/S cells, but the fast current amplitude was smaller for IB4⁺ F/S cells than for the IB4⁻ F/S cells. Because previous studies have shown that the fast current is TTX-sensitive (TTX-S) and the slow current is TTX-R, and because both SNS/PN3 and
15 NaN putatively underlie TTX-R currents in DRG neurons, the properties of the slow currents in IB4⁺ and IB4⁻ cells were examined. The mean midpoints of activation and steady-state inactivation for slow currents were more negative for IB4⁺ than for IB4⁻ cells (Fig. 4C). While these differences are small, they are significant ($p < 0.005$). Figure 4B shows the distribution of inactivation midpoint values in individual IB4⁺ and IB4⁻ cells.

Table 1. Current amplitude: comparison of sodium currents in IB4⁺ and IB4⁻ neurons.

	IB4 ⁺		IB4 ⁻	
	with slow	w/o slow	with slow	w/o slow
<u>0 nM TTX</u>				
fast amplitude (nA)	29±2	---	46±10	31±4
slow amplitude (nA)	31±3	---	32±8	0.7±0.3
% cells	100%	0%	63%	37%
<u>250 nM TTX</u>				
TTX-R amplitude (nA)	38±4	---	37±7	1.0±0.2
% cells	100%	0%	60%	40%

Although the present inventors have previously shown that prepulse subtraction and TTX-subtraction give essentially the same results (Cummins and Waxman, 1997), indicating that all of the slow current is TTX-R and all of the fast current is TTX-S in DRG neurons, Scholz et al. (1998) observed a fast TTX-R current in young DRG neurons (7-21 days), raising the possibility that SNS/PN3 or NaN might encode a fast TTX-R current in DRG neurons. Therefore, sodium currents in IB4⁺ and IB4⁻ neurons in the presence of 250 nM TTX, which blocks 98% of the TTX-S current were also examined. Under these conditions, all IB4⁺ cells (n=30) but only 60% of IB4⁻ neurons (n=30) expressed large (>200 pA/pF) sodium currents. Thus, in agreement with the present inventors' prior classification, DRG neurons studied in the presence of 250 nM TTX can also be subdivided into three groups: IB4⁺ with TTX-R sodium current, IB4⁻ with TTX-R sodium current and IB4⁻ without TTX-R sodium current (Fig. 5A, Table 1). The amplitude of the TTX-R current was similar for the IB4⁺ and IB4⁻ groups that expressed sodium currents.

The rate of inactivation (τ_h) for the TTX-R sodium currents in the IB4⁺ and IB4⁻ cells was compared. τ_h , measured at 0 mV, was significantly ($p < 0.05$) slower for the currents in IB4⁻ cells (6.1 ± 0.7 ms, n=18) than for the currents in IB4⁺ cells (4.6 ± 0.3 ms, n=30). However, while τ_h was longer in IB4⁻ cells than in IB4⁺ cells, all of the TTX-R currents in both groups had time constants greater than 2.5 ms and therefore are considered slow currents. Since all of the currents recorded in the presence of TTX were slow, and since fast TTX-R sodium currents were not observed in either IB4⁺ cells or IB4⁻ cells, the present inventors' data suggest that both NaN and SNS/PN3 encode slow TTX-R currents. It is interesting to note that the percentage of IB4⁻ cells that expressed little or no sodium current in the presence of 250nM TTX was similar to the percentage of IB4⁻ cells that expressed only fast sodium currents in the absence of TTX. Thus, as with previous studies (Kostyuk et al., 1981; McLean et al., 1988; Roy and Narahashi, 1992; Cummins and Waxman, 1997), the data are consistent with the fast currents being solely TTX-S and slow currents being solely TTX-R.

The midpoint of activation and steady-state inactivation of the TTX-R current was significantly ($p < 0.001$) more negative for IB4⁺ neurons than for IB4⁻ neurons that produce TTX-R sodium currents (Fig. 5B). The midpoint of steady-state inactivation ranged from

-31 to -44 mV for TTX-R currents in IB4⁺ cells and from -25 to -36 mV for TTX-R currents in IB4⁻ cells. Fig. 5C shows the distribution of the midpoints of activation and steady-state inactivation for the TTX-R currents. While 50% of IB4⁺ cells have a midpoint of inactivation that is more negative than or equal to -37 mV, none of the IB4⁻ cells do. Conversely, while 39% of the IB4⁻ cells have a midpoint of inactivation that is positive to -31 mV, none of the IB4⁺ cells do. However, while the extremes were dominated by IB4⁺ cells and IB4⁻ cells, respectively, there was overlap between the two groups.

10 **Example 3: *Effect of GDNF and NGF on SNS/PN3 mRNA expression***

The effect of GDNF and NGF on SNS/PN3 mRNA expression was examined in IB4⁺ and IB4⁻ neurons dissociated from adult L4/L5 DRG and cultured for 7 days with normal culture media ("control"), or with media supplemented with NGF, GDNF, BDNF or a combination of NGF and GDNF. DRG neurons dissociated and cultured in this manner, in the absence of exogenously added growth factors, have previously been shown to display changes in levels of sodium channel III and SNS/PN3 mRNAs similar to those seen 7 days after nerve transection *in vivo* (Dib-Hajj et al., 1996; Black et al., 1997). In agreement with a role for GDNF in the maintenance of IB4 reactivity in a subpopulation of small DRG neurons (Bennett et al., 1998b), the IB4 signal intensity was reduced, but discernable, in neurons maintained in cultures for 7 days without exogenously-added GDNF.

In comparison to 7 DIV control neurons, GDNF treatment significantly ($p_c < 0.01$) upregulated SNS/PN3 in both IB4⁺ and IB4⁻ neurons (Fig. 6). The effect of GDNF on SNS/PN3 mRNA expression was significantly ($p_c < 0.001$) more prominent in IB4⁺ neurons (n=74) than in IB4⁻ neurons (n= 51). Similar to GDNF treatment, NGF supplement significantly ($p_c < 0.001$) enhanced the SNS/PN3 hybridization signal in both IB4⁺ (n=91) and IB4⁻ (n=72) neurons compared to IB4⁺ and IB4⁻ 7 DIV control neurons (Fig. 6). However, unlike GDNF treatment, NGF did not preferentially upregulate ($p = ns$) SNS/PN3 in IB4⁻ neurons (n=91) compared to IB4⁻ neurons (n=72) (Fig. 6, 7). The combination of NGF and GDNF significantly increased the SNS/PN3 hybridization signal

in both IB4⁺ and IB4⁻ neurons compared to 7 DIV control neurons; however, the signal in IB4⁺ neurons was less than that observed with either treatment alone, while IB4⁻ neurons showed similar hybridization signals for the neurotrophins alone or in combination (Fig. 6). In contrast, it was observed that BDNF had no effect on SNS mRNA levels.

5

Example 4: *NGF up-regulation of SNS/PN3 mRNA is blocked by K252a*

The up-regulation of SNS/PN3 by NGF could be mediated through the trkA pathway; alternatively, the upregulation may be through a pathway involving the p75 receptor. K252a, in the 100-400 nM range, is a potent inhibitor of NGF action through the

10 trk receptor and has been used to separate effects of NGF mediated through the high-affinity trkA receptor and the low affinity p75 receptor (Kase et al., 1987; Doherty and Walsh, 1989; Tapley et al., 1992; Kahle et al., 1994; Buck and Winter, 1996; De Bernardi et al., 1996). In control experiments without addition of K252a, exogenously-added NGF significantly ($p_c < 0.001$) increased the expression of SNS/PN3 mRNA in DRG neurons

15 maintained in culture for 7 DIV compared to neurons in culture for 7 DIV without added NGF (Fig.8). K252a blocked the effect of NGF on SNS/PN3 expression in a concentration-dependent manner at all concentrations of K252a tested (100- 400nM). The addition of K252a alone did not have a significant effect on SNS/PN3 mRNA expression compared to untreated controls (Fig. 8). These observations indicate that trkA is a

20 necessary component for the effect of NGF on the expression of SNS/PN3 mRNA. Since Schwann cells, which are present in the cultures, express p75 but not trkA (Yamamoto et al., 1993), these results argue that the effect of NGF on SNS/PN3 expression is mediated through a direct action involving TrkA on DRG neurons.

25 **Example 5: *Effect of GDNF and NGF on NaN mRNA***

While NaN expression is decreased following axotomy (Dib-Hajj et al., 1998b) the effect of culturing on NaN mRNA expression has not previously been established. As described for SNS, maintaining of DRG neurons in culture for 7 days without addition of growth factors (n=125) significantly ($p_c < 0.001$) reduced the levels of NaN mRNA

30 compared to freshly dissociated neurons (1 DIV) (n= 95). The reduction was, however,

limited to IB4⁺ neurons, as no significant change was seen in IB4⁻ neurons.

To examine if NaN mRNA is regulated by NGF and GDNF, NaN hybridization signals of DRG neurons treated in culture for 7 days with NGF (n=99), GDNF (n=122), BDNF (n= 93) or a combination of GDNF and NGF (n= 97) were compared to those in control cultures maintained for 7 days without addition of growth factors (n=125). In contrast to the effect on SNS/PN3 mRNA, NGF addition to the culture medium did not alter NaN levels in DRG neurons at 7 DIV in either IB4⁺ (n= 45) or IB4⁻ (n= 54) neurons (Fig. 9,10). In contrast, GDNF supplement to the culture medium significantly ($p_c < 0.001$) increased NaN hybridization signal in IB4⁺ neurons (n= 83) but had no effect on IB4⁻ neurons (n= 39) (Fig. 9,10). These results indicate that GDNF, but not NGF, regulates the expression of NaN mRNA, and are consistent with a direct effect of GDNF on sensory neurons that express receptors for the GDNF-family of neurotrophins. When combining GDNF and NGF, the NaN hybridization was significantly ($p_c < 0.001$) increased in IB4⁺ neurons (n= 57) compared to 7 DIV control neurons, whereas no significant change was seen in IB4⁻ (n= 40) neurons. The hybridization signal in IB4⁺ neurons treated with the combination of GDNF and NGF was somewhat lower than in cultures treated with GDNF alone (Fig. 9). BDNF had no significant effect on the levels of NaN expression compared to control neurons.

20

Example 6: *Effect of GDNF and NGF treatment on TTX-R currents*

Sodium currents in small DRG neurons after 7 DIV in the absence (control) or presence of exogenously added NGF, GDNF or combination NGF and GDNF was examined. For these experiments, IB4-reactivity after each cell was selected for recording was also determined. The staining obtained with FITC-labeled IB4 was much less intense at 7 DIV than that observed at 1 DIV. The intensity of IB4 fluorescence was clearly greater in the GDNF and GDNF/NGF groups than it was in the control and NGF groups at 7 DIV. In the absence of exogenously added GDNF, the reduced fluorescence intensity made it difficult to accurately classify the cells as IB4⁺ or IB4⁻. In contrast, IB4-reactivity in the *in situ* hybridization experiments (see above) was assessed with biotin-labeled IB4

and Cy2-conjugated streptavidin, which provide for enhanced signal amplification. Because of the uncertainty in the IB4-reactivity classification in the patch-clamp experiments on DRG neurons after 7 DIV, sodium current data was not subdivided into IB4⁺ and IB4⁻ cell groups.

5 Sodium currents were recorded in the presence of 250 nM TTX to isolate TTX-R currents. Figure 11A shows representative currents for each of the four groups of neurons. In the control group the amplitude of the TTX-R current was significantly lower (6.1 ± 1.9 nA, $n=41$) than that recorded at 1 DIV (23.9 ± 12.5 nA, $n=36$, Fig. 11B). This reduction in TTX-R currents following *in vitro* axotomy is very similar to that observed following *in*
10 *vivo* axotomy (Cummins and Waxman, 1997). In neurons treated with NGF, the TTX-R current amplitude was similar (6.2 ± 1.5 nA, $n=40$) to that observed in control neurons at 7 DIV. Neurons treated with GDNF, on the other hand, had significantly larger TTX-R currents (13.7 ± 2.4 nA; $n=40$) than that of control neurons at 7 DIV ($p < 0.02$; Fig. 11B). The GDNF/NGF group had the largest peak current amplitude (16.1 ± 2.4 nA, $n=40$),
15 although this was not significantly different from that of the GDNF group. As a measure of TTX-R current expression, the percentage of cells expressing TTX-R current amplitudes > 3 nA (Table II) was determined. In contrast to neurons at 1 DIV, where ~ 90 % of cells displayed TTX-R currents > 3 nA, at 7 DIV less than a quarter of the control cells had TTX-R current amplitudes > 3 nA. GDNF treatment was far superior to NGF
20 treatment in increasing the proportion of cells with large TTX-R currents (Table II).

Because of the extensive neurites that develop in culture, a detailed characterization of the voltage-dependent and kinetic properties of the TTX-R currents in neurons at 7 DIV was not made. However, the TTX-R currents in all four groups appeared slow and the midpoints of activation and steady-state inactivation at 7 DIV were generally
25 similar to those observed for IB4⁺ cells at 1 DIV.

Example 7: *Effect of GDNF and NGF on TTX-R currents in small DRG neurons from SNS-null mice*

Small DRG neurons were taken from SNS-null mice (Akopian et al., 1999) and
 5 cultured *in vitro* using the methods described for rat neurons above. Neurons were
 cultured in the presence of 250 nM TTX and further in the presence or absence of GDNF
 (10ng/ml) or NGF (50 ng/ml). The currents were elicited by 200 ms test pulses to
 potentials ranging from -80 to +40 mV in 5 mV steps. Cells were held at -100 mV. As
 shown in Figures 12A, in untreated cultures the persistent non-SNS TTX-R sodium
 10 current traces were drastically decreased after 7 days in vitro (7 DIV). By contrast, in
 GDNF-treated cultures the TTX-R currents were maintained at control levels. Similarly,
 persistent non-SNS TTX-R peak currents were returned to control levels by the addition of
 GDNF, as shown in Figure 12B. Notably, the addition of NGF to the culture medium did
 not significantly effect the size of persistent non-TTX-R current compared to the control 7
 15 DIV neurons.

Example 8: *GDNF attenuates the decrease of TTX-R current density and persistent Na⁺ currents in vivo*

Uninjured neurons predominantly express slow-inactivating TTX-R and slowly-
 20 repriming TTX-S Na⁺ currents. Following axotomy, TTX-R current density is greatly
 reduced and rapidly repriming TTX-S currents predominate. Consequently, the effect *in*
vivo of exogenously administered GDNF delivered to transected nerves on sodium
 currents in small DRG neurons was examined. GDNF (1.2 µg/day per animal) was
 delivered to DRG neurons *in vivo* via an osmotic pump attached to the transected sciatic
 25 nerve. (Dib-Hajj et al. 1998a). The opposite sciatic nerve was also transected and hooked
 to a pump containing only Ringer's solution.

As shown in Figures 13A and 13B, both slowly-inactivating and persistent TTX-R
 currents were partially restored to toward control levels in GDNF-treated axotomized

neurons. It was also observed that TTX-S currents in GDNF-treated axotomized neurons expressed intermediate repriming kinetics. Thus, GDNF treatment can mitigate the effects of axotomy on the sodium currents of a sub-population of small DRG sensory neurons.

- 5 Although the present invention has been described in detail with reference to the examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety.

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CLAIMS

1. A method to treat pain or hyperexcitability phenomena in an animal or human subject by administering an amount of GDNF that is effective to alter TTX-R Na⁺ current flow through NaN sodium channels in DRG or trigeminal neurons.
2. The method of claim 1, wherein the sensory neuron is a DRG or trigeminal neuron.
3. A method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of GDNF capable of at least partially restoring the normal balance between various types of TTX-R and TTX-S sodium channels in sensory neurons.
4. The method of claim 3, wherein the sensory neuron is a DRG or trigeminal neuron.
5. The method of claim 3 or 4, wherein the TTX-R sodium channels are selected from the group consisting of SNS/PN3 and NaN channels.
6. A method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of modulating the transcription or translation of mRNA encoding sodium channels selected from the group consisting of SNS/PN3 and NaN channels.
7. The method of claim 6, wherein the agent is a neurotrophin.
8. The method of claim 6, wherein the neurotrophin is selected from the group

consisting of NGF and GDNF and or other members of their families.

9. The method of claim 6, wherein the agent modulates the production or activity of a neurotrophin that modulates the activity of the sodium channel.

5

10. The method of claim 9, wherein the agent modulates the level or activity of GDNF or NGF.

11. A method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of altering the transcription or translation of mRNA encoding the NaN sodium channel.

12. A method of identifying an agent which modulates TTX-R Na⁺ current through NaN channels comprising the step of:

15 determining whether the agent alters or modulates the expression of GDNF or at least one biological activity of GDNF.

13. The method of claim 10, wherein the agent modulates the GDNF induction of NaN.

20

14. A cell that has been transformed to express a functional recombinant GDNF receptor and, optionally, recombinant NaN.

15. A method to screen candidate compounds for use in treating pain and hyperexcitability phenomena comprising the steps of exposing the cell to the compound in the presence or absence of GDNF and determining the resultant level of expression or activity of the cell's Na⁺ channels.

16. The method of claim 15, wherein the cell is the transformed cell of claim 12.

17. The method of claim 15, wherein the Na⁺ channel is selected from the group consisting of the SNS/PN3 and NaN channels.

5 18. The method of claim 15, wherein the cell does not express SNS.

19. The method of claim 15, wherein the cell is present in a living animal.

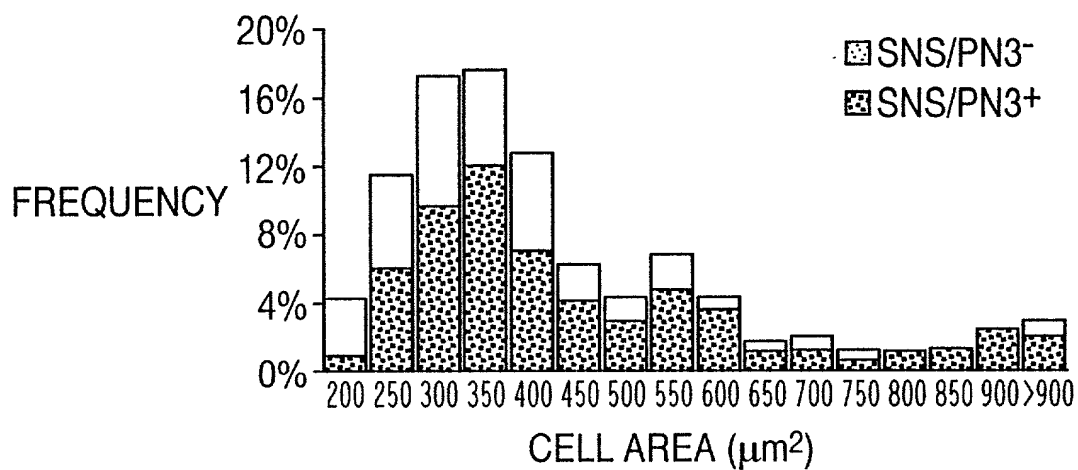
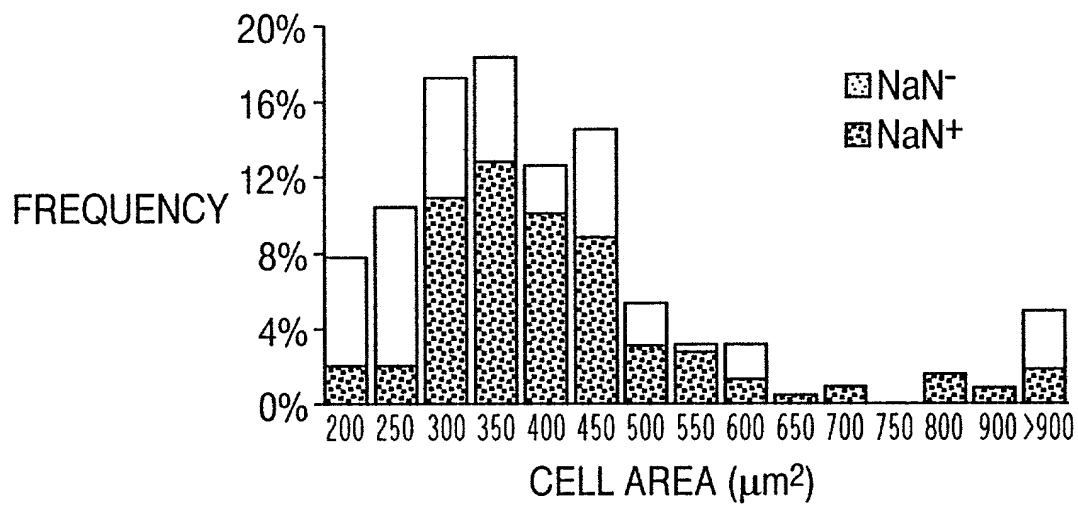
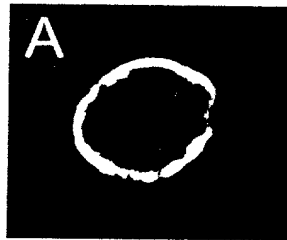
FIG. 1A**FIG. 1B**

FIG. 2A

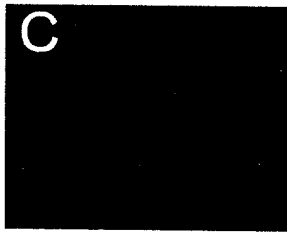


B



FIG. 2B

FIG. 2C



D



FIG. 2D

FIG. 2E



F



FIG. 2F

FIG. 2G



H



FIG. 2H

FIG. 3A

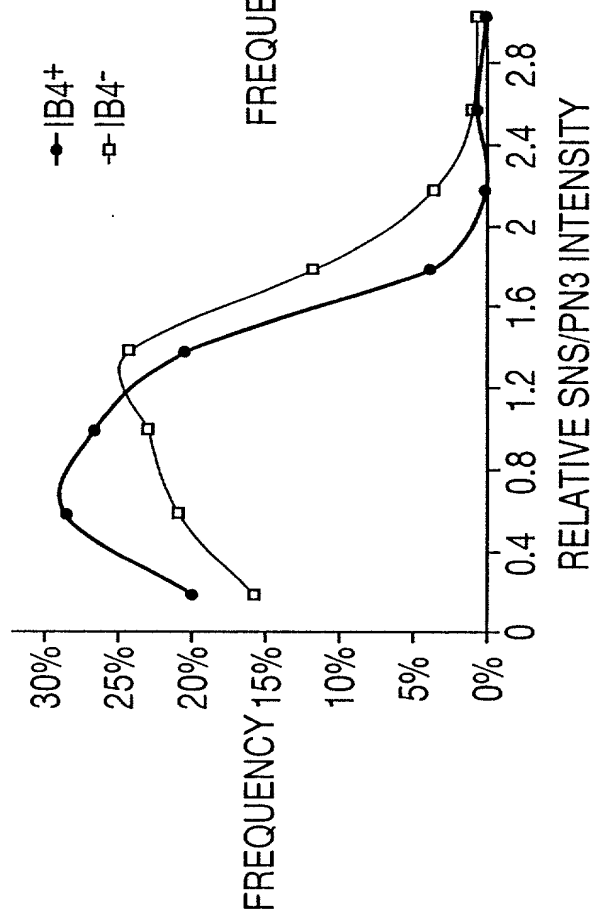


FIG. 3B

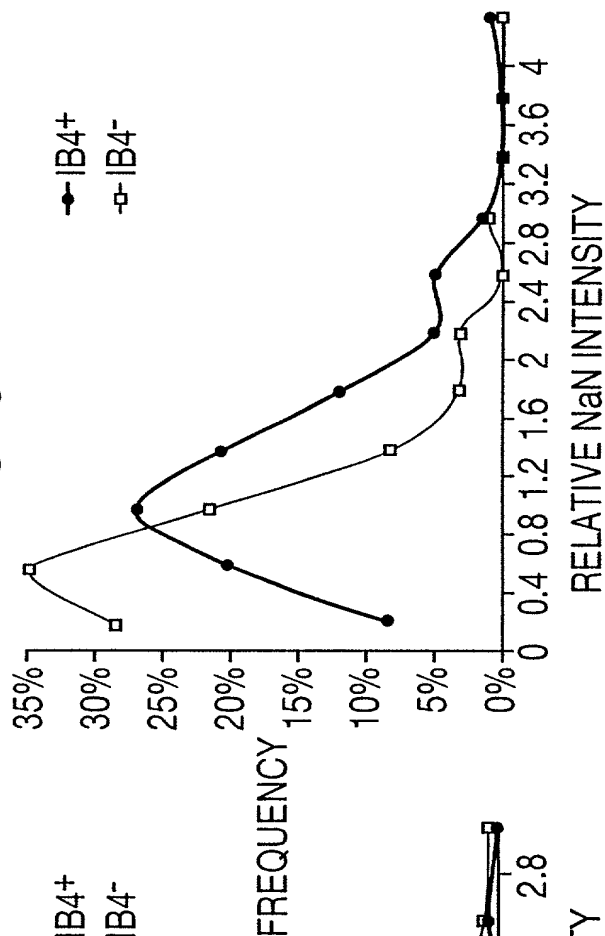


FIG. 4A

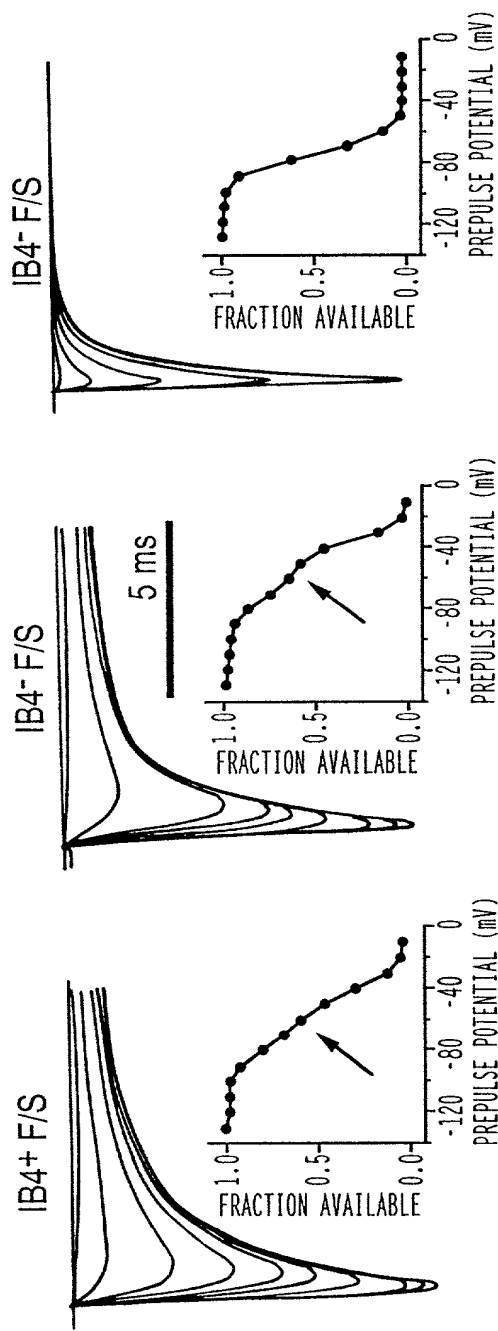


FIG. 4C

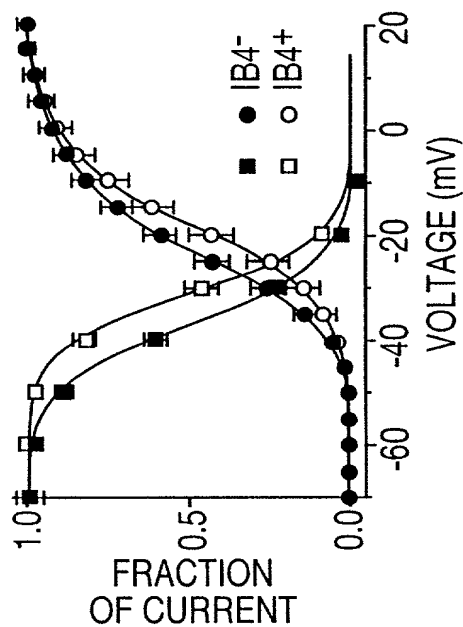


FIG. 4B

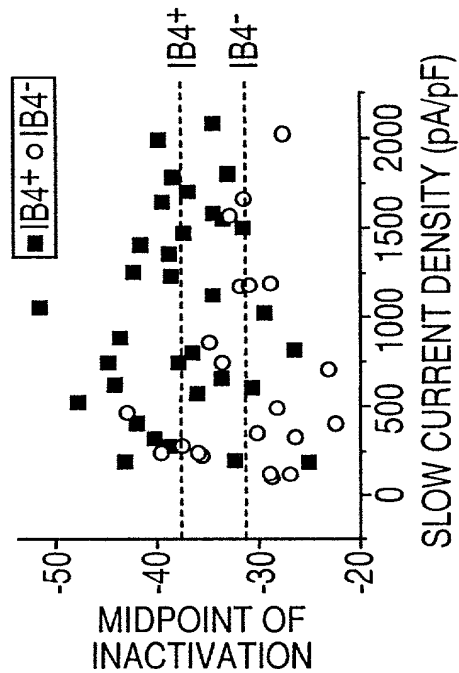


FIG. 5A

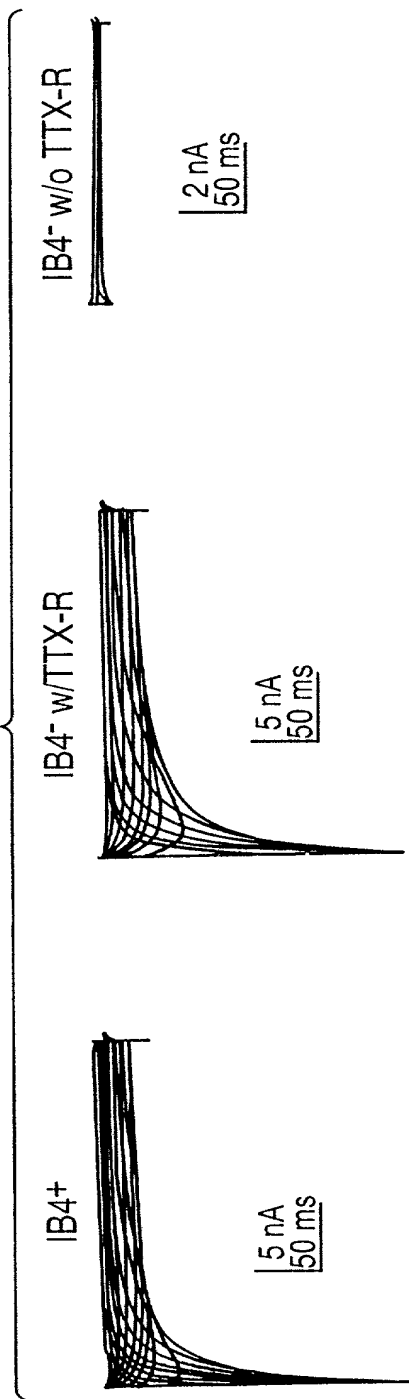


FIG. 5B

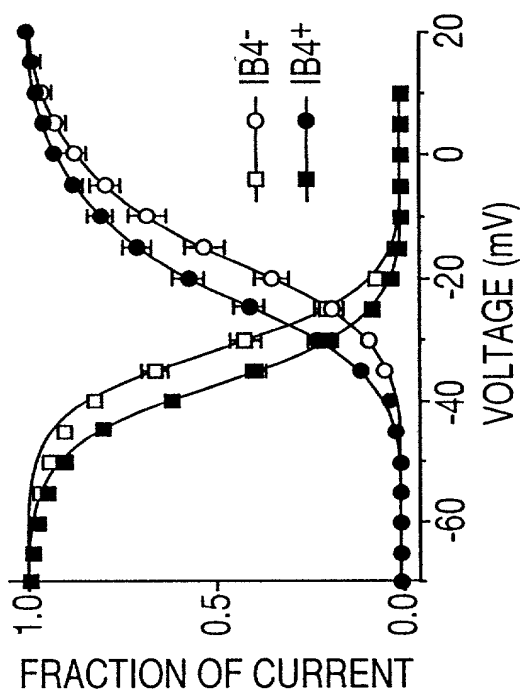


FIG. 5C

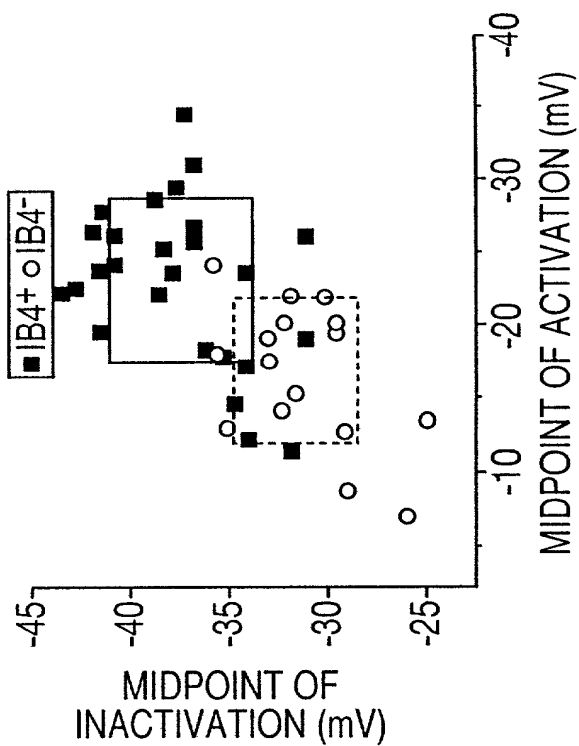


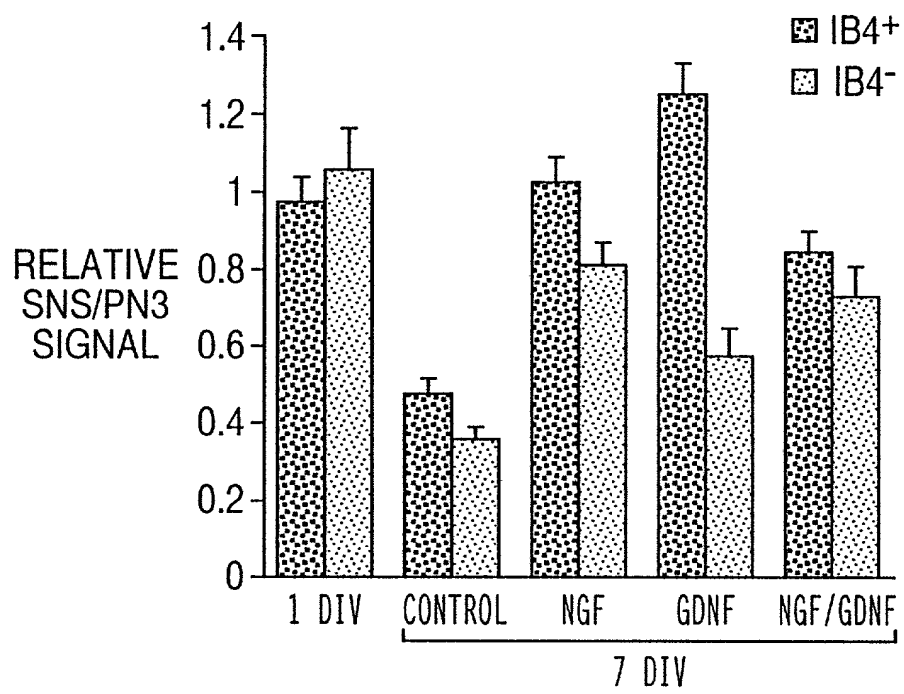
FIG. 6

FIG. 7A A



B



FIG. 7B

FIG. 7C C



D



FIG. 7D

FIG. 7E E



F



FIG. 7F

09/856274: FIG. 7

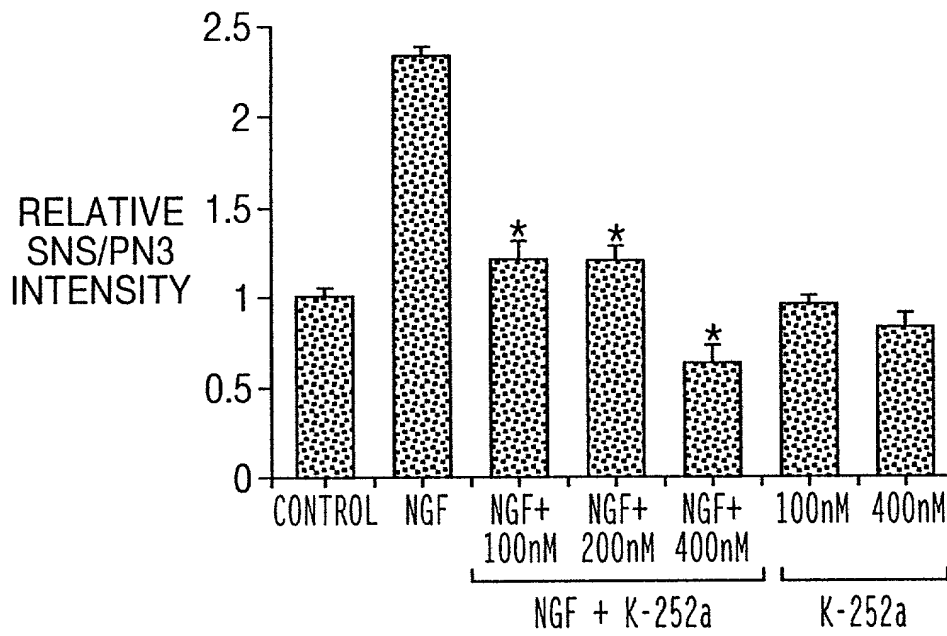
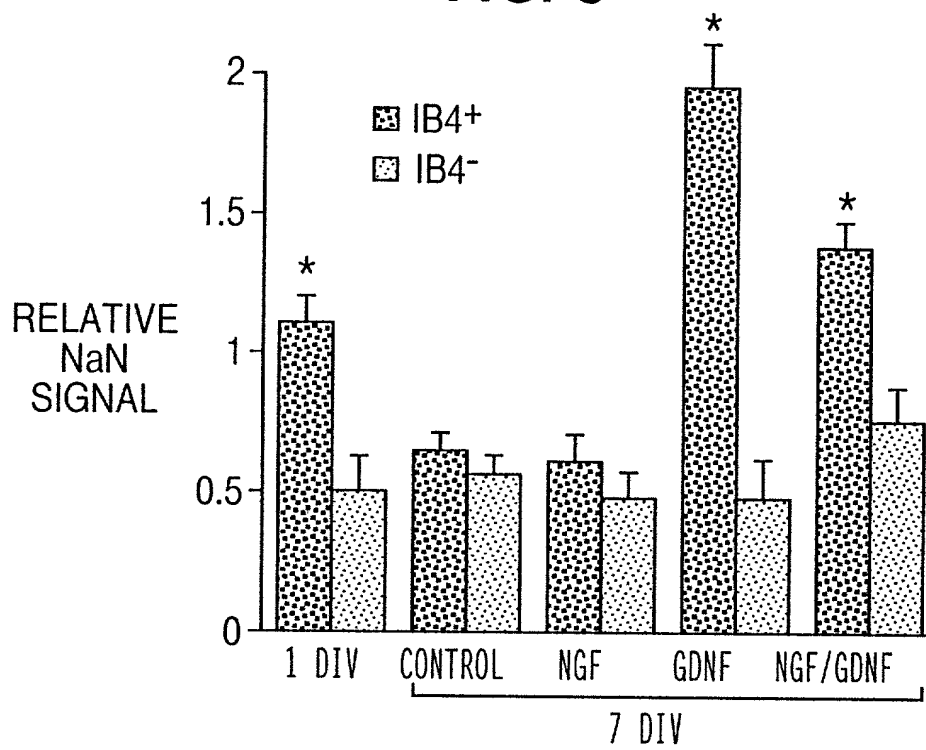
FIG. 8

FIG. 9

A



C



E



FIG. 11A

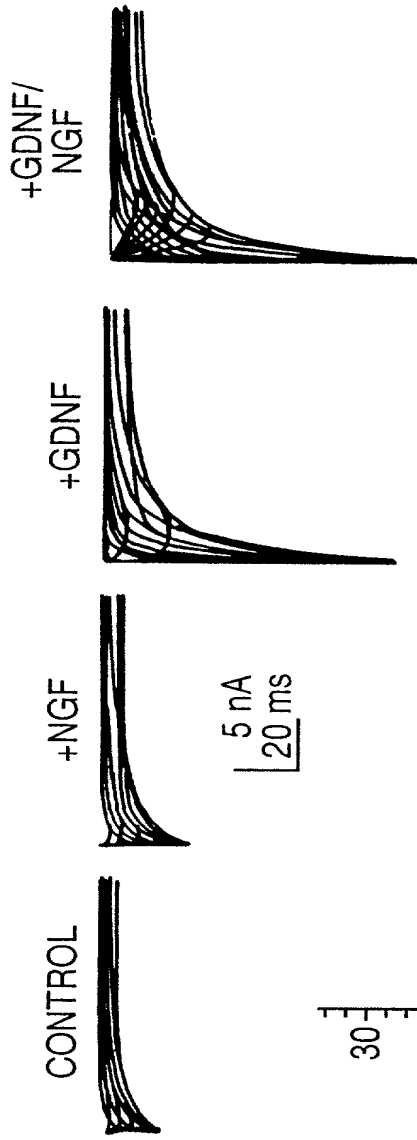


FIG. 11B

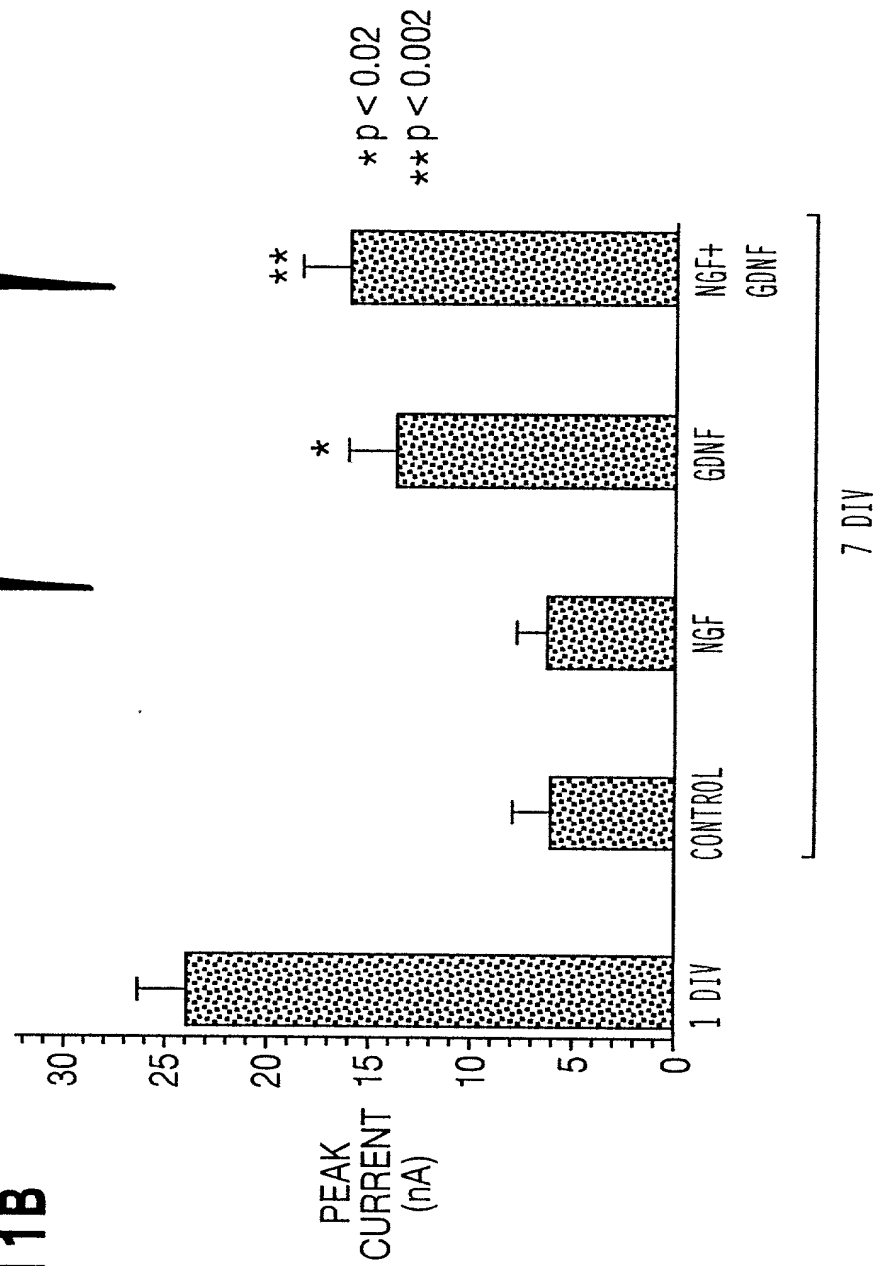


FIG. 12A

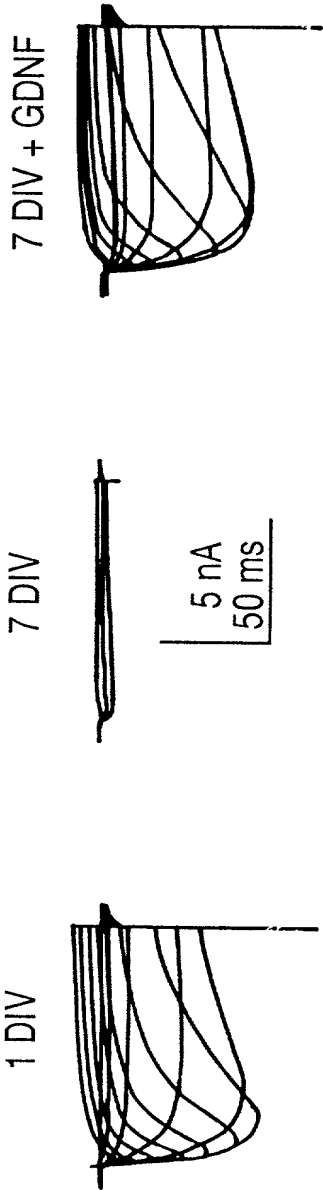


FIG. 12B

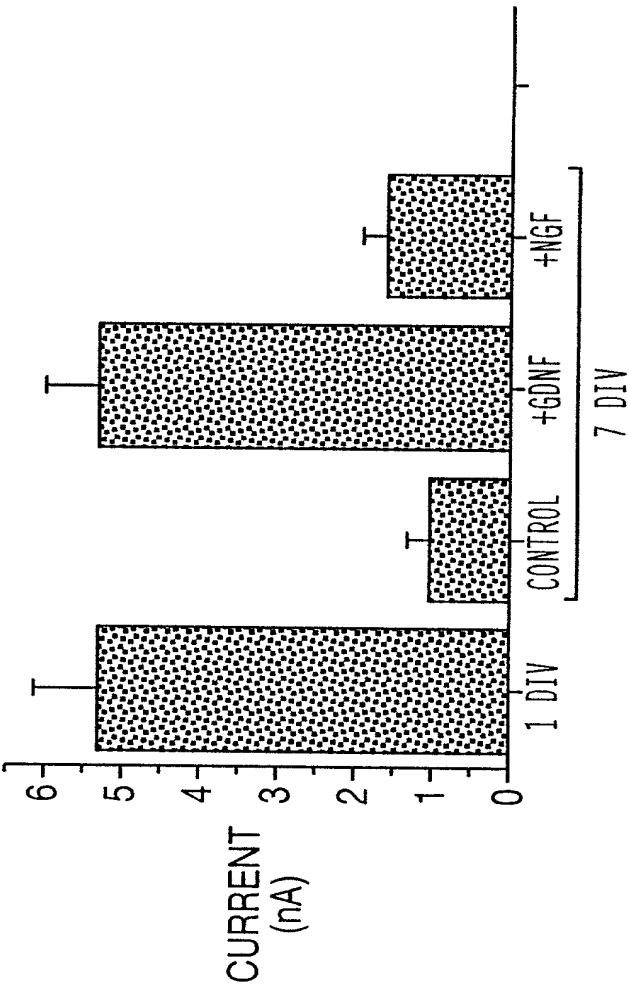


FIG. 13A

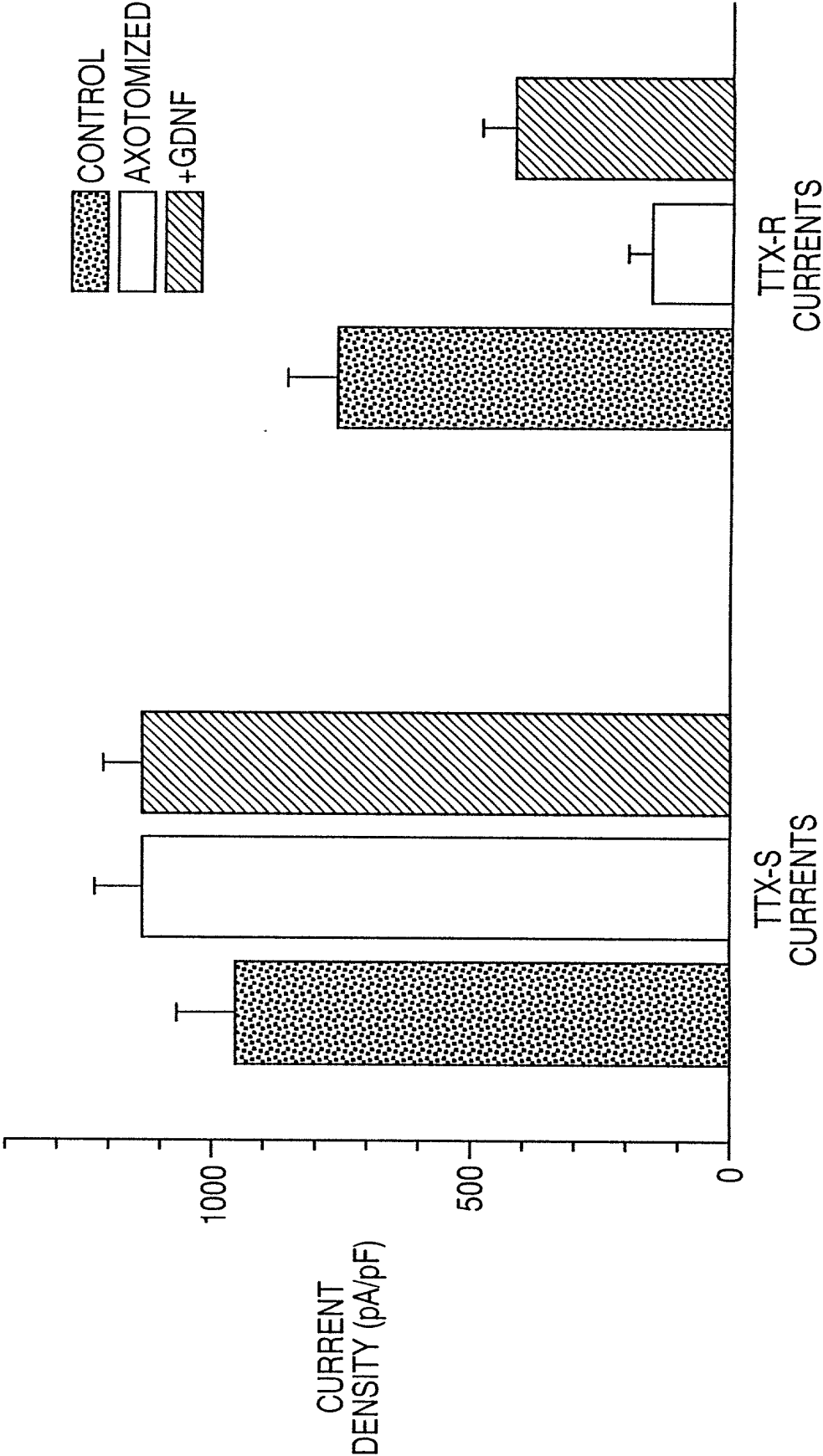
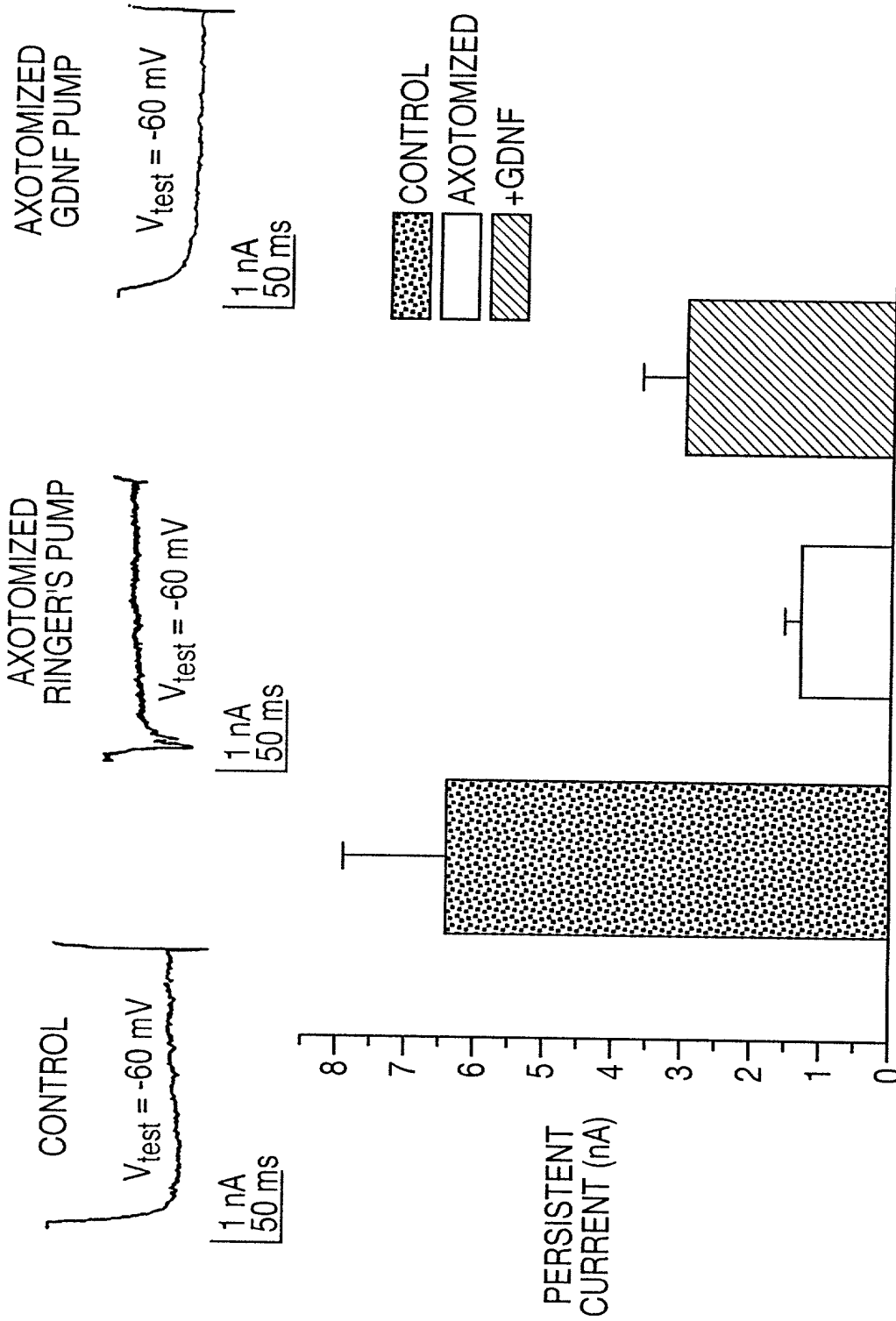


FIG. 13B



COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

U.S. Department of Commerce
Patent and Trademark Office

Attorney Docket **044574-5044-US**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

EFFECTS OF GDNF AND NGF ON SODIUM CHANNELS IN DRG NEURONS

was filed as PCT international application Number PCT/US99/27368 on November 19, 1999 and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office information which is material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign applications for patent or inventor's certificate or § 365(a) of any PCT international applications designating at least one country other than the United States of America listed below and have also identified below any foreign applications for patent or inventor's certificate or any PCT international applications designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the applications of which priority is claimed:

PRIOR FOREIGN APPLICATIONS

COUNTRY	APPLICATION NUMBER	DATE OF FILING	PRIORITY CLAIMED
			<input type="checkbox"/> Yes <input type="checkbox"/> No

I hereby claim the benefits under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

U.S. PROVISIONAL APPLICATIONS

U.S. PROVISIONAL APPLICATION SERIAL NO.	U.S. FILING DATE
60/109,666	November 20, 1998

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States applications or § 365(c) of any PCT international applications designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior applications in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior applications and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT

U.S. APPLICATIONS		STATUS (CHECK ONE)		
U.S. APPLICATION SERIAL NO.	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint the registered practitioners of Morgan, Lewis & Bockius LLP included in the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number.
Customer Number: 009629

Direct Telephone Calls To:
Reid G. Adler
202-467-7000

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF SOLE INVENTOR	Sulayman D. Dib-Hajj	
----------------------------	----------------------	--

RESIDENCE	East Lyme, CT	CITIZENSHIP: United States
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POST OFFICE ADDRESS	110 Lovers Lane, East Lyme, CT 06333	
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SOLE INVENTOR'S SIGNATURE	DATE
---------------------------	------

FULL NAME OF SECOND INVENTOR	Jenny Fjell Hejlmstrom <i>Hejlmstrom JF 10/15/01</i>	
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RESIDENCE	Stockholm, Sweden	CITIZENSHIP: Sweden
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POST OFFICE ADDRESS	Vestmannag. 44, 4tr 113 25 Stockholm JF 10/15/01 Valhallav.150A 2TR, SE-115 24 Stockholm Sweden	
---------------------	--	--

SECOND INVENTOR'S SIGNATURE	DATE
-----------------------------	------

FULL NAME OF THIRD INVENTOR	Joel A. Black	
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RESIDENCE	Hamden, CT	CITIZENSHIP: United States
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THIRD INVENTOR'S SIGNATURE	DATE
----------------------------	------

FULL NAME OF FOURTH INVENTOR	Stephen G. Waxman	
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RESIDENCE	New Haven, CT	CITIZENSHIP: United States
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POST OFFICE ADDRESS	333 Cedar Street LCI 707, New Haven, CT 06510	
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FOURTH INVENTOR'S SIGNATURE	DATE
-----------------------------	------

Listing of Inventors Continued on Attached Page ☒ Yes ☐ No

Combined Declaration For Patent Application and Power of Attorney - (Continued)
(includes Reference to PCT International Applications)

Attorney Docket 044574-5044-US

FULL NAME OF FIFTH
INVENTOR

Theodore R. Cummins

RESIDENCE

East Haven, CT

CITIZENSHIP: United States

POST OFFICE ADDRESS

61 Catherine Street, East Haven CT 06512

FIFTH INVENTOR'S SIGNATURE

DATE

Listing of Inventors Continued on Attached Page ☐ Yes ☒ No

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

U.S. Department of Commerce
Patent and Trademark Office

Attorney Docket **044574-5044-US**

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PRIOR FOREIGN APPLICATIONS

COUNTRY	APPLICATION NUMBER	DATE OF FILING	PRIORITY CLAIMED
			<input type="checkbox"/> Yes <input type="checkbox"/> No

I hereby claim the benefits under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

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U.S. PROVISIONAL APPLICATION SERIAL NO.	U.S. FILING DATE
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PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT

U.S. APPLICATIONS		STATUS (CHECK ONE)		
U.S. APPLICATION SERIAL NO.	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint the registered practitioners of Morgan, Lewis & Bockius LLP included in the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number.

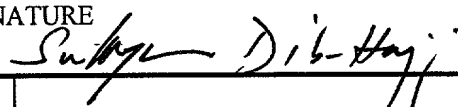
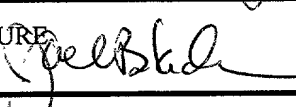
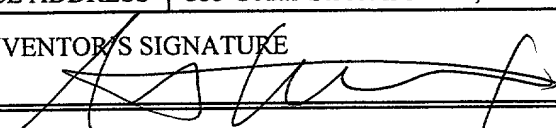
Customer Number: 009629

Direct Telephone Calls To:

Reid G. Adler

202-467-7000

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF SOLE INVENTOR	Sulayman D. Dib-Hajj 1-00	
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SOLE INVENTOR'S SIGNATURE		DATE 6/20/01
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RESIDENCE	New Haven, CT CT	CITIZENSHIP: Sweden
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SECOND INVENTOR'S SIGNATURE		DATE
FULL NAME OF THIRD INVENTOR	Joel A. Black 3-00	
RESIDENCE	Hamden, CT CT	CITIZENSHIP: United States
POST OFFICE ADDRESS	51 Berkeley St 208 Dorrance Street, Hamden, CT 06518	
THIRD INVENTOR'S SIGNATURE		DATE 6/26/01
FULL NAME OF FOURTH INVENTOR	Stephen G. Waxman 4-00	
RESIDENCE	New Haven, CT CT	CITIZENSHIP: United States
POST OFFICE ADDRESS	333 Cedar Street LCI 707, New Haven, CT 06510	
FOURTH INVENTOR'S SIGNATURE		DATE 6/20/01

Listing of Inventors Continued on Attached Page ☒ Yes ☐ No

Combined Declaration For Patent Application and Power of Attorney - (Continued) (includes Reference to PCT International Applications)		Attorney Docket 044574-5044-US
FULL NAME OF FIFTH INVENTOR	Theodore R. Cummins <i>5-02</i>	
RESIDENCE	East Haven, CT <i>CT</i>	CITIZENSHIP: United States
POST OFFICE ADDRESS	61 Catherine Street, East Haven CT 06512	
FIFTH INVENTOR'S SIGNATURE	DATE	
<i>Theodore R. Cummins</i>	<i>6/27/01</i>	
Listing of Inventors Continued on Attached Page <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No		

044574-5044-US